## About the author

Henry Muriuki Kariithi was born 18-09-1976 in Nyeri, Kenya. In 2001, he graduated *cum laude* with a BSc degree in Biochemistry (University of Nairobi, Kenya), and in 2009, he obtained an MSc degree in Cellular and Molecular Biotechnology (Wageningen University, The Netherlands) sponsored by a WU MSc Scholarship. In September 2010, he started his PhD at the Laboratory of Virology, Wageningen University, on a WU PhD Fellowship, and in March 2011, he transferred to NUFFIC PhD Sandwich Program. As part of his Sandwich PhD, Henry worked at the Joint FAO/IAEA Program of Nuclear Techniques in Food and Agriculture, IPCL Seibersdorf, Austria, to study infectomics of *Glossina* hytrosavirus and explore methods to control the virus in tsetse mass-production facility at Seibersdorf. Henry also spent part of his PhD study time as a Research Assistant at his home institute, the Kenya Agricultural Research Institute (KARI) where, in addition to his research on *Glossina* hytrosavirus, he worked on epidemiology and diagnostics of arboviruses, specifically Rift Valley fever virus. His research at Wageningen University's Virology, IAEA's Seibersdorf and KARI Biotechnology Laboratories resulted to this thesis. Henry looks forward to assume his research duties at KARI, which is being newly reorganized under the Kenya Agricultural Research Organization (KARO). Henry enjoys history, music, scrabble, and nature walks.

*Glossina* hytrosavirus control strategies in tsetse fly factories

Henry M. Kariithi 2013

# *Glossina* hytrosavirus control strategies in tsetse fly factories

## Application of infectomics in virus management

Henry M. Kariithi

## Invitation

You are cordially invited to attend the defense of my PhD thesis entitled:

***Glossina* hytrosavirus control strategies in tsetse fly factories:**

**Application of infectomics in virus management**

The ceremony will be held at the Aula of Wageningen University, General Foulkesweg 1A, Wageningen on Wednesday, November 20th 2013 at 4pm.

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# Propositions

1. Hytrosaviruses cannot be used as bio-control agents for tsetse flies.
   (this thesis)

2. As an insect host metamorphoses to adulthood, vertical transmission of an insect virus becomes epizootically more important than horizontal transmission.
   (this thesis)

3. Microbial symbiosis is a remarkable source of evolutionary innovation.

4. The contents of a proteome, the repertoire of domains in the protein sequences, can be used to trace the evolutionary history of an organism.

5. The presence of protein-coding regions or open reading frames in a genome does not necessarily imply the presence of functional proteins.

6. The impression that hard work is degrading to fashionable life does not appeal to reason.

7. The maxim that 'a slave' first loses his name and then adopts the lingo is true in all facets of life.
   (Inference from '*Mũrogi wa kagogo*', a satire by Ngũgĩ wa Thiong'o, 2006)

Proposition belonging to the PhD thesis

**_Glossina_ hytrosavirus control strategies in tsetse fly factories:**

**Application of infectomics in virus management**

Henry Muriuki KARIITHI

Wageningen, 20 November 2013

# *Glossina* hytrosavirus control strategies in tsetse fly factories:

## Application of infectomics in virus management

**Henry Muriuki Kariithi**

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This research was conducted under the auspices of the Graduate School of Production Ecology and Resource Conservation.

# *Glossina* hytrosavirus control strategies in tsetse fly factories:

## Application of infectomics in virus management

**Henry Muriuki Kariithi**

**Thesis**

submitted in partial fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
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H.M. Kariithi

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*To Graham, Jochebed and Leona*

# Abstract

African trypanosomosis is a fatal zoonotic disease transmitted by tsetse flies (Diptera; Glossinidae); blood-sucking insects found only in sub-Saharan Africa. Two forms of trypanosomoses occur: the animal African trypanosomosis (AAT; nagana), and the human African trypanosomosis (HAT; sleeping sickness). Since there are no effective vaccines against trypanosomosis, tsetse fly eradication is the most effective disease control method. Tsetse flies can be effectively eradicated by the sterile insect technique (SIT), which is applied in an area-wide integrated pest management approach. SIT is an environmentally benign method with a long and solid record of accomplishments. SIT requires large-scale production of sexually sterilized male flies (by exposure to a precise and specific dose of ionizing radiation, usually from a $^{60}$Co or $^{137}$Ce source), which are sequentially released into a target wild insect population to out-compete wild type males in inseminating wild virgin females. Once inseminated by sterile males, the virgin females do not produce viable progeny flies. Importantly, these females do not typically re-mate. Ultimately, the target wild insect population can decrease to extinction. However, tsetse SIT programs are faced with a unique problem: laboratory colonies of many tsetse species are infected by the *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV; family *Hytrosaviridae*). GpSGHV-infected flies have male aspermia or oligospermia, underdeveloped female ovarioles, sterility, salivary gland hypertrophy syndrome (SGH), distorted sex ratios, and reduced insemination rates. Without proper management, symptomatic GpSGHV infections (characterized by SGH symptoms) can cause collapse of *Glossina* colonies. To ensure colony productivity and survival, GpSGHV management strategies are required. This will ensure a sustained supply of sterile males for SIT programs. The aim of this PhD research was to investigate the functional and structural genomics and proteomics (infectomics) of GpSGHV as a prerequisite to development of rationally designed viral control strategies. A series of experiments were designed to: (i) investigate epidemiology and diversity of GpSGHV; (ii) identify GpSGHV proteome and how viral and host proteins contribute to the pathobiology of the virus; and (iii) investigate the interplay between GpSGHV, the microbiome and the host, and how these interactions influence the outcomes of viral infections. By relating GpSGHV and host infectomics data, cost-effective viral management strategies were developed. This resulted in significant reduction of GpSGHV loads and elimination of SGH from laboratory colonies of *G. pallidipes.*

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# Chapter 1

# General introduction

## Introduction

This PhD dissertation covers various aspects of the pathobiology, epidemiology, morphology and morphogenesis of the *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV), a double-stranded DNA (dsDNA) virus classified in the *Hytrosaviridae* family of insect viruses (ICTV; Abd-Alla *et al.*, 2009b). GpSGHV is a major pathogen of laboratory colonies of *Glossina* spp. (Diptera; Glossinidae). Typically, a small proportion of laboratory *G. pallidipes* flies infected by GpSGHV develop hypertrophied salivary glands and midgut epithelial cells, and show gonadal/ovarian anomalies, distorted sex ratios, reduced insemination rates, fecundity and lifespan. These symptoms are rarely observed in wild tsetse fly populations.

In East Africa, *G. pallidipes* is one of the most important vectors of the debilitating zoonotic disease, African trypanosomosis. A large arsenal of tsetse and trypanosomosis management tactics is available. The sterile insect technique (SIT) is a robust and effective method to eradicate tsetse fly populations when integrated with other control tactics in an area-wide integrated approach. SIT requires production of sterile male flies in large-scale tsetse production facilities. To supply sufficient numbers of sterile males for the SIT component against *G. pallidipes*, strategies must be developed to manage GpSGHV infections in the fly colonies. This chapter provides a historic account of tsetse fly and trypanosomoses control, and a chronology of the emergence and biogeography of hytrosaviruses. The thesis rationale is also described.

## African trypanosomoses – the "neglected tropical diseases"

Tsetse flies are important vectors of two debilitating diseases; human African trypanosomosis (HAT or sleeping sickness), and animal African trypanosomosis (AAT or Nagana) (Mattioli *et al.*, 2004). Tsetse flies and trypanosomosis render vast areas of agricultural land un-exploitable, especially during the rainy seasons (Mamoudou *et al.*, 2008). Although there are over 30 species and sub-species of tsetse, most of which can transmit trypanosomosis, only 8-10 of these species are of medical and agricultural importance. The most important tsetse vectors are the riverine species (*G. palpalis*, *G. fuscipes*, and *G. tachinoides*) in Western and Central Africa and the savannah species

(*G. morsitans*, *G. austeni* and *G. pallidipes*) in Eastern and Southern Africa (Smith *et al.*, 1998). Although tsetse fly fossils have been found in 26-million-year-old shales of Florissant, Colorado, USA (Cockerell, 1907), to date, tsetse flies are confined to Africa except from an isolated population on the Arabian Peninsula (Elsen *et al.*, 1990).

HAT is one of the most serious of the so-called 'neglected tropical diseases' (NTDs) (Hotez and Kamath, 2009). NTDs are a group of chronic diseases endemic in low-income populations in Africa, Asia and the Americas (Hotez *et al.*, 2007). Trypanosomosis is restricted to 37 sub-Saharan African countries and its distribution extends to more than 10 million square kilometres of the African continent (Cecchi *et al.*, 2008) (**Figure 1**).

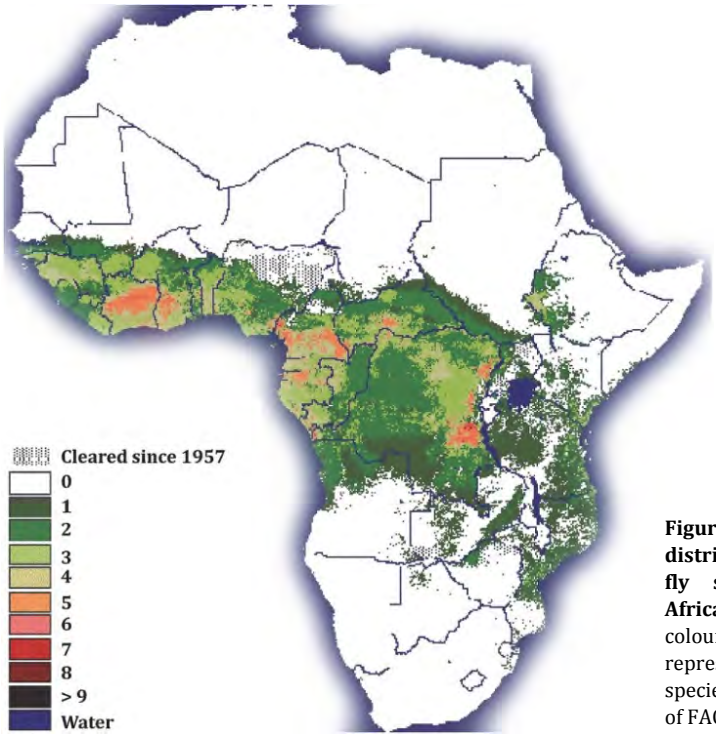


**Figure 1: Representation of the distribution of different tsetse fly species in sub-Saharan African countries.** The different colours in the figure legend represent the different tsetse fly species in the map (Map courtesy of FAO).

The people at the highest risk of tsetse fly bites and of contracting HAT are the rural populations that primarily depend on small-scale agriculture, fishing, animal husbandry and hunting. Resurgence and epidemics of HAT are often associated with economic decline, civil disturbances/wars, population movements and refugees (Smith *et al.*, 1998). The presence of tsetse and trypanosomosis is considered as one of the "roots of hunger and poverty" in sub-Saharan Africa (Vreysen, 2006). It is

estimated that approximately 90% of Africa's livestock consists of herds in small villages (Otte and Chilonda, 2003). This implies that maintaining healthy animals can make the difference between subsistence misery and an acceptable lifestyle for the farmers and their families. FAO estimates that ~ US$ 4.75 billion worth of agricultural products are lost annually due to AAT (including ~3 million cattle deaths), and ≥ 100 human lives are lost daily due to HAT.

African trypanosomoses are difficult to treat, and there are no effective vaccines available against both forms of the disease. None of the available trypanocidal drugs for HAT is ideal; their treatment schedules are prolonged, excruciatingly painful (often described by patients as "fire in the veins") and requires continuous hospitalization (Matovu *et al.*, 2001). Generally, trypanosomes initiate their lifecycle by first colonizing the tsetse hosts' midguts, and then migrate into the ectoperitrophic space, and to the salivary glands via the alimentary canal and the mouthparts (Oberholzer *et al.*, 2010). The parasites differentiate into the final mammalian-infective trypomastigocyte in the tsetse salivary glands, and are then transmitted to the mammalian host by an infected tsetse bite (Sharma *et al.*, 2009). It should however be noted that some steps in the lifecycle of trypanosomes are group-specific. For instance, members of the *Trypanosoma vivax* group only stay in the proboscis; the *T. congolense* group has a cycle involving the proboscis and the midguts; while only the *T. brucei* group has a cycle involving the midgut and the salivary glands (Vickerman, 1985).

Without treatment, African trypanosomoses can be fatal, but the fatalities differ from one group of trypanosomes to another. For instance, in West Africa *T. b. gambiensis* causes a chronic disease that can take many years to kill a patient, while in East Africa, *T. b. rhodiensis* causes an acute disease that can kill a patient within weeks of the infective tsetse fly bite (Brun *et al.*, 2010; Chappuis *et al.*, 2005). The most widely used drug, melarsoprol, which is used to treat the CNS forms of trypanosomes, was developed in 1949 (Friedheim, 1949); the drug is toxic as it causes vomiting and kidney damage, and 5-10% of patients die from the treatment itself (Burri *et al.*, 2000; 2010). It is also important to note that, one of the biggest problems in the treatment of HAT is that the patients are usually so weak that they are more likely to die from the treatment rather than from the disease. In addition, patients need to be properly fed for several weeks to regain strength before the commencement of treatments. This presents a very serious problem considering that there are hardly any available funds to properly feed. There are reports of increasing drug-resistance and drug counterfeiting (Barrett *et al.*, 2011; Geerts *et al.*, 2001).Therefore, control of the disease vector (tsetse) is of critical importance, and probably represents the most sustainable trypanosomosis control method (Schofield and Kabayo, 2008).

# An overview of tsetse fly control methods

## *Why tsetse flies are suited for eradication*

Two main characteristics of the tsetse fly render them suitable for eradication. Firstly, compared to other insects of medical and agricultural importance, tsetse flies have low dispersal and reproduction rate (*k* -strategists) (Leak, 1998). Tsetse flies are viviparous, and typically produce 8-10 offspring in their lifespan (Attardo *et al.*, 2006). Therefore, unlike many insect vectors that produce large numbers of eggs (*r* -strategist), tsetse flies have limited capacity to rebound in areas where their populations have been reduced. Secondly, tsetse flies are adapted to exploit stable habitats provided by vertebrate nests or human dwellings with low levels of crossbreeding. This means that tsetse flies have reduced genetic variability within each vector population and therefore, have limited capacity to respond through selection pressure to various control interventions (Dujardin and Schofield, 2004).

## *The 'evolution' of tsetse fly control methods*

Tsetse control methods have evolved from discriminate bush clearing and wild game culling at the beginning of the 20$^{th}$ Century, to the widespread applications of broad-spectrum insecticides after the Second World War (Allsopp, 2001). However, these control measures have negative impacts on the environment and ecosystems, and are not compatible anymore with today's environmental requirements. The overall effect of bush clearing and game culling is loss of biodiversity. The use of insecticides raises concerns on the fate of non-target organisms (du Toit, 1954). Besides, some insecticides persist long in the environment, and the residues of insecticides end up in water bodies where they endanger aquatic organisms, or are transferred and concentrated up the food chains (Grant, 2001). To respond to the negative environmental and ecological impacts, advancements were made in using traps, insecticide-impregnated targets (Brightwell and Dransfield, 1997) and live bait technologies (Rowlands *et al.*, 2001). Although these methods have succeeded in reducing the local tsetse populations (Leak, 1998), each method has its own limitations. Firstly, the methods do not protect the cleared areas from re-invasion by tsetse flies from residual pockets and from neighbouring territories (Brightwell *et al.*, 1997). Secondly, the methods are applied in administratively defined regions and run for an administratively specified time (Schofield and Kabayo, 2008), which mostly depend on how long external donor funds are available for the projects. Since the methods are cannot be sustained beyond the time of the external donor-funded projects, the risk of the cleared areas to be re-infested by tsetse flies from the neighbouring (uncleared) areas increases. In the light of these developments, there was need to explore other methods for tsetse and trypanosomosis control.

# Application of the sterility principle for tsetse eradication

## *The discovery of the sterile insect technique for control of insect pests*

In 1937, Knipling developed the theory of controlling insect pest populations by manipulating their reproductive capacity. He likewise modelled that a target insect population could be eradicated when the release of sterile males was applied on an area-wide basis against an entire insect pest population in a delineated area (Knipling, 1955; 1959), rendering them sterile. This method, commonly known as the sterile insect technique (SIT), involves large-scale production of the insects in mass-rearing facilities. Excess male flies are sexually sterilized by exposure to a precise and specific dose of ionizing radiation, usually from a $^{60}$Co or $^{137}$Ce source (Robinson, 2002; 2005). The sterile males are then sequentially released into the target insect population in numbers that allows them to out-compete wild males for mating with wild virgin females (Abila *et al.*, 2003). After the virgin females have mated with the sterile males, embryogenesis is arrested, and consequently no viable offspring is produced. When the release of the sterile males is sustained, the size of the target insect population will decline and can eventually become extinct.

## *How successful is SIT in the eradication of insect pests?*

The SIT is a robust control tactic that has been used very successfully against insect pests that are important for agriculture and trade. For instance, the SIT was used to control the screwworm fly *Cochliomyia hominivorax* (Diptera; Cochliomyia) from the Southern USA, Mexico, Central America and Panama (Wyss, 2000) and from northern Libya after a serious outbreak in 1989 (Lindquist *et al.*, 1992). The SIT was also used to eradicate Mediterranean fruit fly *Ceratitis capitata* (Diptera; Ceratitis) populations in Chile, Mendoza (Argentina), Mexico etc., and in Central America, South Africa, Israel etc., respectively (Enkerlin, 2005; Franz, 2005). Lately, the SIT has also been used with great success against several Lepidopteran pests such as the codling moth *Cydia pomonella* (L.) in the Okanagan Valley of Canada (Bloem *et al.*, 2006a; 2006b), the false codling moth *Thaumatotibia leucotreta* (Meyrick) in South Africa, the Australian painted apple moth *Teia anartoides* (Walker) in New Zealand (Vreysen *et al.*, 2007), and the pink bollworm *Pectinophora gossypiella* (Saunders) in Texas, New Mexico, Arizona, California (USA) and in Sonora and Chihuahua of northern Mexico (Enkerlin, 2005; Koyama *et al.*, 2004).

## *The control (eradication) of tsetse flies by SIT using the AW-IMP approach*

The SIT has also played a pivotal role in the sustainable area-wide eradication of the tsetse fly *Glossina austeni* from Unguja Island (Zanzibar) (Vreysen *et al.*, 2000). This program was preceded by successful applications of the technique against

*G. palpalis gambiensis* and *G. tachinoides* in the Sideradougou area in Burkina Faso and against *G. palpalis palpalis* in the Lafia area of Nigeria (Oladunmade *et al.*, 1990; Politzar *et al.*, 1984). The programs in Burkina Faso and Nigeria were however not implemented according to area wide integrated pest management (AW-IPM) principles and the tsetse-cleared area was re-invaded by tsetse flies after the programs were completed. Following the area-wide eradication of *G. austeni* the island was declared tsetse-free in 1997; it still is to-date.

### *The Glossina SGHV: A hindrance to tsetse fly control in sub-Sahara Africa*

The success of SIT in eradicating *G. austeni* and trypanosomosis from Unguja Island inspired African Governments to call for increased efforts to control tsetse flies and trypanosomoses on mainland Africa. Consequently, an AW-IMP program with an SIT component was initiated in 1997 to eradicate *G. pallidipes* from a 25, 000 square kilometres of under-utilized fertile land in the Southern Rift Valley of Ethiopia. For the Ethiopian SIT program, laboratory tsetse colonies were established at Insect Pest Control Laboratory (IPCL) of the Joint FAO/IAEA Agriculture and Biotechnology Laboratories in Seibersdorf, Austria, and at Kality, Ethiopia (Feldmann *et al.*, 2005). However, efforts to establish mass-rearing of tsetse colonies revealed that *G. pallidipes* colonies are vulnerable to a virus infection that causes salivary gland hypertrophy syndrome (SGH), and leads to reduction in productive fitness in male and female tsetse flies (Abd-Alla *et al.*, 2010b). The virus infection results in significant reduction in fertility and fecundity leading to decline of the colonies within a few generations (Kariithi *et al.*, 2013a).

## Discovery and distribution of hytrosaviruses

### *Hytrosaviruses in Dipteran insects*

Chronological developments from the first emergence of SGH to the discovery of the hytrosaviruses causing the syndrome are summarized in **Table 1**. Whitnall reported the first description of SGH symptoms in *G. pallidipes* in the 1930's (Whitnall, 1932; 1934) during investigations of *Trypanosoma*-infections in Zululand, South Africa. SGH was later reported to be sex-linked, and to favour development of trypanosomes in *G. morsitans* (Burtt, 1945). In the 1970's, SGH was associated with a virus found in cytoplasmic vacuoles of the salivary glands and midgut epithelial cells of *G. fuscipes* and *G. m. morsitans* (Jenni, 1973; 1976; 1974a; 1974b). The virus was at that time described as "virus-like particles" (VLPs), morphologically resembling viral particles that had been described in *Drosophila*, *Aedes aegypti*, and in nematodes. Since other hematophagous insects (e.g. mosquitoes, ticks, sand flies and gnats) had been widely known to transmit arboviruses, the *Glossina* virus was erroneously suggested to be an arbovirus. Other features of the *Glossina* virus that led to this conclusion is the shape,

size of the viral particles, and the fact that arboviruses were known to replicate in the host salivary glands (Chamberlain, 1968; Janzen *et al.*, 1970; Mims *et al.*, 1966), followed by release of mature viral particles via saliva during feeding (La Motte, 1960). A resemblance of the tsetse fly virus to baculovirus was also suggested based on their extended rod-shape morphology (Jaenson, 1978b). Mating experiments revealed that the virus is transmitted from the mother to her progeny and not from the male parent (Jaenson, 1986).

**Table 1:** Chronological history of the discovery and distribution of salivary gland hytrosaviruses (SGHVs).

| Investigator(s) | Year | Major contribution(s) | References |
|---|---|---|---|
| Whitnall | 1932, 34 | First published record of SGH *Glossina* spp. | (Whitnall, 1932; 1934) |
| Burtt | 1937, 45 | Suggested that SGH is sex-linked | (Burtt, 1945) |
| Jenni *et al.,* | 1973, 74, 76 | Described virus particles in *G. morsitans* and *G. fuscipes fuscipes*; suggested Golgi-ER viral assembly | (Jenni, 1973; 1976; 1974a; 1974b) |
| Lyon | 1973 | First published record of SGH in *M. equestris* | (Lyon, 1973) |
| Jaenson | 1978 | First clear association of viral particles with SGH | (Jaenson, 1978b) |
| Amargier *et al.,* | 1979 | Reported SGH in *M. equestris* | (Amargier *et al.*, 1979) |
| Otieno *et al.,* | 1980 | Reported SGH as common feature in wild *G. pallidipes* | (Otieno *et al.*, 1980) |
| Opiyo | 1983 | Reported poor productivity of *G. pallidipes* colony at Kenya Trypanosomiasis Research Institute (KETRI), Kenya | (Opiyo and Okumu, 1983) |
| Odindo *et al.,* | 1981, 83, 86 | Demonstrated that viral particles are infectious *per os*; First report that *Glossina* virus has dsDNA genome | (Odindo *et al.*, 1981; 1982; 1986; 1986) |
| Jaenson | 1986 | First report on reduced insemination rates, fecundity and lifespan in laboratory colonies of *G. pallidipes* | (Jaenson, 1986) |
| Ellis *et al.,* | 1987 | Reported SGH in Zimbabwe and Ivory Coast | (Ellis and Maudlin, 1987; Gouteux, 1987) |
| IAEA | 1987, 89 | Reported poor productivity of *G. pallidipes* colonies at Insect Pest Control Laboratories (IPCL), Seibersdorf, Austria | |
| Odindo | 1988 | Proposed *Glossina* virus as a bio-control agent | (Odindo, 1988) |
| Jura *et al.,* | 1988, 89, 92, 93 | Demonstrated transmission of *Glossina* virus after artificial infection | (Jura *et al.*, 1988; 1993; 1992; 1989) |
| Kokwaro *et al.,* | 1990-91 | Cytopathology of virus particles in tsetse salivary glands | (Kokwaro *et al.*, 1990; 1991) |
| Shaw | 1993 | Reported SGH in *G. m. Swyenatoni* and *G. brevipalpis* | (Shaw and Moloo, 1993) |
| Coler *et al.,* | 1993 | First published record of SGH in *M. domestica* | (Coler *et al.*, 1993) |
| Sang *et al.*, | 1996-99 | Reported SGHV in tsetse milk glands, mid-gut and male accessory reproductive glands | (Sang *et al.*, 1996; 1997; 1998; 1999) |
| IAEA | 2002 | Collapse of an Ethiopian-derived *G. pallidipes* colony at IPCL, Seibersdorf, Austria | |
| Kokwaro | 2006 | Reported virus particles in male accessory reproductive glands of *G. m. morsitans* Westwood | (Kokwaro, 2006) |
| Abd-Alla *et al.;* Garcia-Maruniak *et al.,* | 2008 | *G. pallidipes* and *M. domestica* SGHVs genome sequenced | (Abd-Alla *et al.*, 2008; 2009b) |
| Abd-Alla *et al.,* | 2009 | Establishment *Hytrosaviridae* family | (Abd-Alla *et al.*, 2009b) |
| Salem *et al.,* | 2009 | Transcription analysis of *M. domestica* SGHV | (Salem *et al.*, 2009) |
| Kariithi *et al.,* | 2010-13 | Described the proteome, ultra-structure and morphogenesis of *Glossina* virus | (Kariithi *et al.*, 2013b; 2010) |
| Prompiboon *et al.,* | 2010 | Reported wild-wide distribution of SGHV in *M. domestica* | (Prompiboon *et al.*, 2010) |
| Luo and Zheng | 2010 | SGHV-like virus described in accessory gland filaments of the parasitic wasp *D. Longicuadata* | (Luo and Zeng, 2010) |
| Boucias *et al.*, | 2013 | Described the role of endosymbionts on trans generational trans mission of SGHV in *G. pallidipes* | (Boucias *et al.*, 2013b) |
| Abd-Alla *et al.,* | 2013 | Reported successful management of GpSGHV and eradication of SGH in *G. pallidipes* colonies | (Abd-Alla *et al.*, 2013) |

In exceptional cases, asymptomatic parents produced symptomatic (SGH-positive) progenies, suggesting that the virus was possibly reactivated from 'latency' by a combination of stress and genetic factors. High prevalence of SGH (15.6%) in wild *G. pallidipes* populations was thought to indicate that SGH contributed to natural regulation of tsetse populations in the field (Odindo, 1982). SGH was reported to occur twice as frequent in males than females, expressed in one-third of the flies that had been fed or micro-injected the virus suspension as tenerals (newly- emerged and non-fed flies), and to cause female sterility, and male aspermia and/or oligospermia (Odindo *et al.*, 1981). It was not until 1986 that the virus causing SGH in *G. pallidipes (*Austen) was discovered to be a novel DNA virus that could not be placed in any of the existing taxa of insect DNA-viruses (Odindo *et al.*, 1986). After purifying the virus by a series of sucrose gradient centrifugations, the virus particles were described as long, non-enveloped rods containing linear double stranded (ds) DNA and 12 different polypeptides (Odindo *et al.*, 1986).

The second description of SGH was reported in adult populations of the narcissus bulb fly, *Merodon equestris* (Diptera; Syrphidae) in the 1970's in southern France (Amargier *et al.*, 1979; Lyon, 1973; Lyon and Sabatier, 1973). The incidence of SGH was reported in 31% and 51% of *M. equestris nobilis* and *M. equestris transversalis* respectively. Degeneration of male and female reproductive organs was also described as the main disease symptom. Similar to the virus causing SGH in tsetse flies, the virus particles in *M. equestris* had ultra-structural features similar to some baculoviruses, and were therefore assumed to be related to tsetse hytrosavirus. To date, no further research has been performed on the bulb fly virus to substantiate this claim.

Coler *et al*., (Coler et al., 1993) reported the third discovery of an SGH in the 1990s in adult house flies, *Musca domestica* (Diptera; Muscidae) during a survey of parasitic nematodes in the fly at a dairy farm in central Florida, USA. SGH and total suppression of oogenesis were described as the main symptoms of the housefly virus infection (Coler *et al.*, 1993). However, unlike in tsetse fly virus, there was no evidence of vertical transmission of the housefly virus from mother to the progeny (Geden *et al.*, 2008; Lietze *et al.*, 2007). *M. domestica* was later confirmed to be naturally infected with the housefly virus, and the virus has been shown to be globally distributed with detections in samples from Africa, North America, Europe, Asia, the Caribbean, and the south-western Pacific (Geden *et al.*, 2011b; Prompiboon *et al.*, 2010). The housefly virus was also reported to replicate in a laboratory colony of the stable fly, *Stomoxys calcitrans* (Diptera; Muscidae), which occurs sympatrically with the house fly, albeit without the classical SGH syndrome (Geden *et al.*, 2011a).

### *Other (potential) members of the hytrosavirus group*

Recently, a virus similar to the hytrosavirus group was accidentally discovered in the accessory gland filaments (AGFs) of the braconid wasp *Diachasmimorpha longicuadata* (Hymenoptera; Braconidae) in a sample derived from a population originally from Hawaii, released in Thailand and introduced to China as a bio-control for the fruit fly, *Bactrocella dorsalis* (Diptera; Tephritidae) in South China (Luo and Zeng, 2010). The wasp AGFs appeared hypertrophied with ultra-structural features similar to hytrosaviruses (Luo and Zeng, 2010). It is tempting to speculate that the discovery of SGH symptoms in the AGFs of *D. longicuadata* is an indication that there are other SGHVs that are yet to be identified. Consequently, it can be concluded that the current shortage of field-based insect virologists, combined with the SGHVs' intrinsic properties (i.e. chronic covert infection of adult stages) may have hindered discoveries of other hytrosaviruses.

## Pathology of hytrosaviruses

Dipteran adult flies infected by hytrosaviruses show gross signs of overt SGH, hence the name SGHVs. (Abd-Alla *et al.*, 2010b; Lietze *et al.*, 2007). Pathological effects of SGHVs have been observed more profoundly in laboratory-bred tsetse fly colonies as well as in the house flies. In 1979, wild-caught *G. pallidipes* from Kibwezi forest in Kenya were used to initiate a colony at the Kenya Trypanosomiasis Research Institute (KETRI). Within two years of its establishment, the colony declined due to poor productivity (Opiyo and Okumu, 1983). A similar trend was noted in another *G. pallidipes* colony established at the Insect Pest Control Laboratories (IPCL) in Seibersdorf, Austria, which experienced a steady decline, eventually leading to its collapse in 2002 (Abd-Alla *et al.*, 2007a; 2010b). Investigations revealed that 85% of the males and 70% of the females had SGH. Tsetse with SGH exhibit discoloured salivary glands that are enlarged 4 times larger than the normal thickness (**Figure 2 A**).

Although there are no obvious external signs of infected flies (Odindo, 1982), hypertrophied glands appear as a pale outline in the male fly's abdomen with irregular ridges on the cuticle (**Figure 2 B**). The discolouration is probably due to the extension of gland cells towards the cell lumina, resulting to constricted gland lumens. The enlarged and chalky-white glands have also been observed in virus-infected house flies (**Figure 2 C and D**) (Coler *et al.*, 1993). The collapse of *G. pallidipes* colonies was ascribed to low productivity due to male testicular degeneration and female ovarian abnormalities caused by the *Glossina* virus (Jura *et al.*, 1988; Kokwaro, 1986; Kokwaro and Murithi, 1988; Kokwaro and Odhiambo, 1981; Sang *et al.*, 1998; 1999).

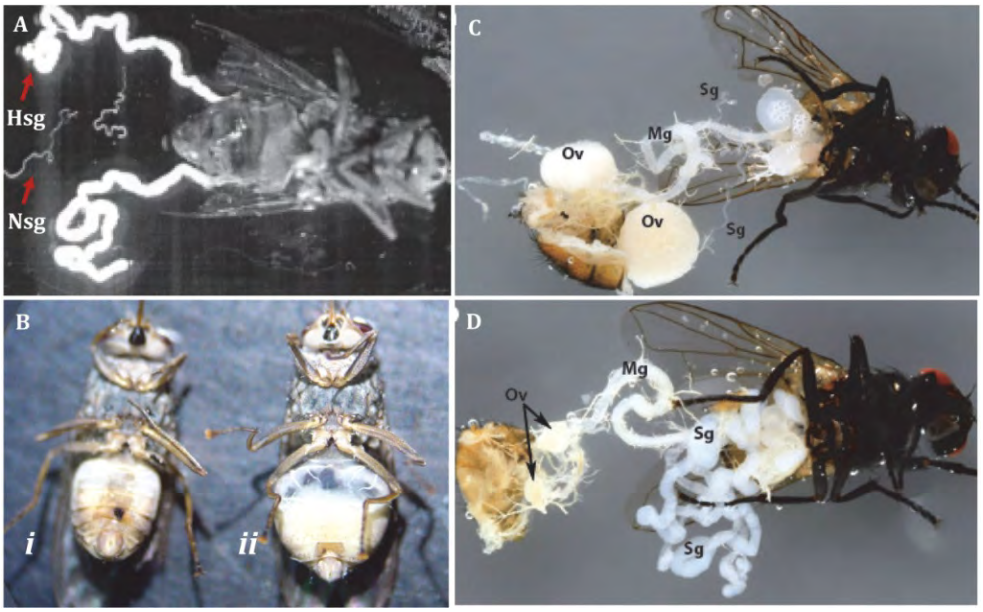


**Figure 2: The pathology of hytrosaviruses.** (A) Normal (Nsg) and hypertrophied (Hsg) salivary glands dissected from *G. pallidipes*. It should be noted that the pair of Nsg is dissected from a different fly for comparison with the Hsg. Notice that the glands exhibiting SGH are enlarged more than four times the size of normal glands; (B) Male *G. pallidipes* with asymptomatic (*i*) and symptomatic (*ii*) salivary glands. (C) Female *M. domestica* with healthy and (D) hypertrophied salivary glands showing lack of ovarian development in the virus-infected fly (D). Abbreviations: Mg, midgut; Ov, ovary; Sg, salivary gland. (Figure sources: Panel A (Abd-Alla *et al.*, 2007a); panel B (Abd-Alla *et al.*, 2009a); panels C and D (Lietze *et al.*, 2011b)).

# *Glossina* hytrosavirus as tsetse fly bio-control agent

## *The potential of Glossina SGHV as a bio-pesticide*

Many insect-pathogenic viruses such as baculoviruses are effective bio-control agents against insect pests (Harrison and Hoover, 2012; Moscardi, 1999). Odindo (1988) first proposed the potential of the *Glossina* virus as a male sterility factor in tsetse fly control. After micro-injection of the virus into laboratory-bred *G. pallidipes*, infection was observed in 13.3% and 30.0% of treated male and female parents, respectively. The prevalence of SGH in the $F_1$ progeny adults was higher than in the parents (80% in males and 58.3% females). Whereas all infected females were fertile, all infected males had SGH and were sterile. Maternal larviposition, $F_1$ pupae weight and $F_1$ pupae incubation periods were normal regardless of the treatments. Two other studies reported that virus-infected males showed reduced reproductive potential (Sang *et al.*, 1999). Based on these results, it was hypothesized that the *Glossina* SGHV may be

applied as a tsetse fly bio-control agent. The hypothesis was that the sterile male parents might compete with normal wild males in mating, and the fertile but infected females might transmit the SGH syndrome trans-ovarially to subsequent generations, since such females produce only infected progeny (Jaenson, 1986), where males are "born" sterile.

### *Why Glossina SGHV cannot be used as a bio-pesticide*

Despite the proposal described above i.e. that *Glossina* SGHV can is a potential bio-control agent, application of the *Glossina* SGHV as bio-pesticide for tsetse fly control is technically challenging. Firstly, recent findings show that neither micro-injection nor *per os* infection of the virus in *G. pallidipes* result in SGH in the same (parental) generation, rather, the syndrome is only detectable in the third (~65%) and fourth (~100%) larviposition cycles of the $F_1$ generation (Boucias *et al.*, 2013b). Secondly, new evidence shows that high prevalence of SGH reduces the mating propensity and competitiveness of males thus affecting the stability and performance of tsetse colonies (Mutika *et al.*, 2012), and hence difficulties in producing high numbers of infected insects. Thirdly, *in vitro* mass production of the virus for field applications is currently impossible due to limitations such as the absence of a cell culture system permissive to the virus. Besides, attempts to produce the virus in an alternative host (house fly) - with short life cycle and easy to produce *en masse* - have been unsuccessful. Fourthly, there is no available evidence for horizontal transmission of *Glossina* SGHV through contact between flies, mating, or faecal contamination, thus limiting the modes of how the virus would be dispersed in the field. Finally, the virus does not produce occlusion bodies, as for instance baculoviruses do to achieve prolonged stability in the environment outside of the host: recent evidence shows that *Glossina* SGHV is highly unstable outside of the host (Kariithi *et al.*, 2013b), with more than 80% of virus suspension losing infectivity after three days at 4°C (Abd-Alla *et al.*, 2011b). Formulation of virus suspensions allowing virus to retain infectivity under both laboratory and field conditions appears insurmountable for the time being. This is in contrast to the *Musca* virus infection system: intra-hemocoelic injection with very low dosage of the virus induces both 100% incidence of SGH, and a total shut down of oogenesis within 72-96 h post-injection (Geden *et al.*, 2011a; Lietze *et al.*, 2007; Lietze *et al.*, 2012). The use of the *Glossina* SGHV as tsetse bio-control agent appears impractical.

## Genome organization of hytrosaviruses

The negative impacts of *Glossina* SGHV infections on laboratory-bred *G. pallidipes* prompted researches to understand the viral biology and pathology. Hypertrophied salivary glands were dissected from *G. pallidipes* flies originating from Tororo, Uganda in 1975, colonized initially at Leiden University, The Netherlands, and subsequently

transferred to IPCL, Seibersdorf, Austria in 1982. The genome of the virus purified from the dissected glands was fully sequenced (NC_010356.1) (Abd-Alla *et al.*, 2008). A total of 322 non-overlapping open reading frames (ORFs) were identified, of which 160 ORFs were presumed to encode putative viral proteins (Abd-Alla *et al.*, 2008). Detection of two bands (super coiled and relaxed forms) after agarose gel electrophoresis of purified DNA, and a lack of end-labelling of the undigested DNA suggested a circular viral genome (**Figure 3**).

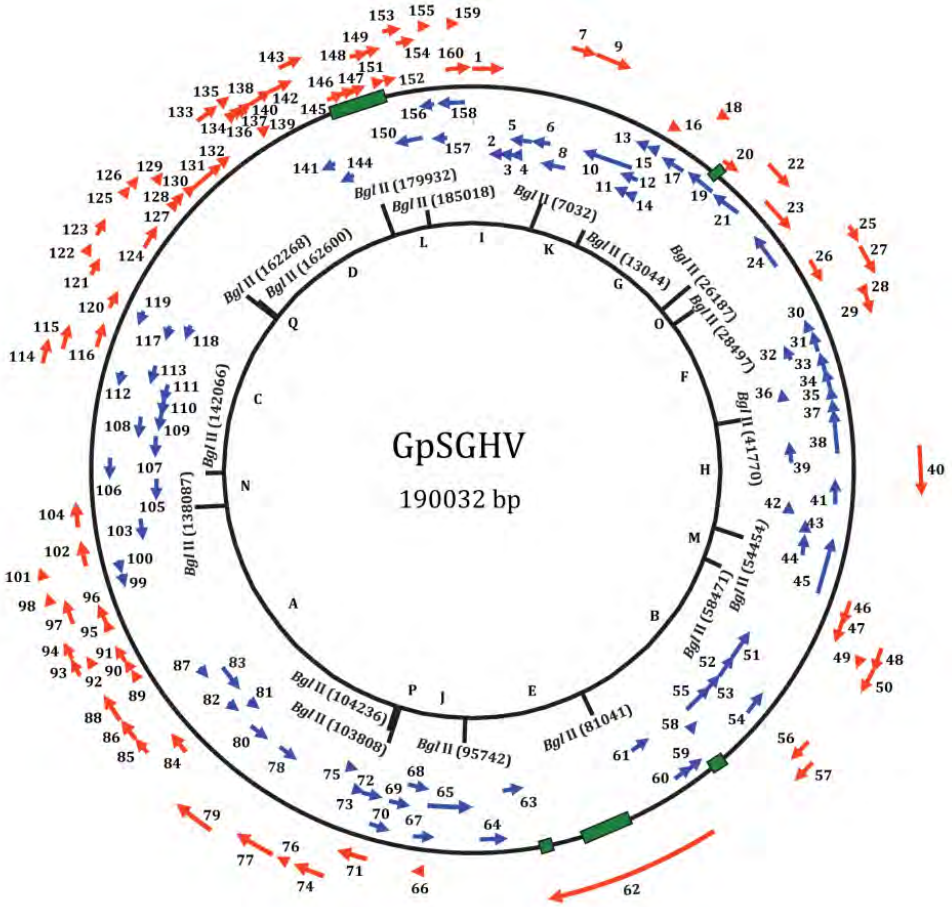


**Figure 3: Circular representation of *Glossina* SGHV genome**. Arrows indicate position and direction of transcription for the potential ORFs. GpSGHV ORF numbers and putative genes are shown. The alphabetical numbers represent restriction fragments generated by BglII enzyme during the electrophoretic profiling of the virus genome.

One hundred thirteen (70.6%) of the 160 ORFs did not match to any of the sequences available in various databases (Abd-Alla *et al.*, 2008). Thirty-seven ORFs (23.1%) were homologues to genes of other viruses, while ten (6.3%) were homologues to non-viral/cellular genes. Most notable of the *Glossina* virus ORFs that were homologues to other viral genes were five of the *per os* infectivity factor genes (*pifs*) (*p74*, *pif-1*, *pif-2*, *pif-3*, and *odv-e66*) encoded by baculoviruses and nudiviruses (Song *et al.*, 2008). Other notable homologies included homologues to sixteen entomopoxvirus and poxvirus genes, three iridovirus and nimavirus genes each, two ascovirus genes and one herpesvirus gene. Most notable of the cellular gene homologues include chitinase, DNA helicases, thymidylate synthases, and several homologues to bacterial genes (Abd-Alla *et al.*, 2008). Approximately 3% of the viral genome contains one inverted repeat (*ir*) sequence and fourteen direct repeat sequences (*drs*) composed of 51-246 bp units.

As mentioned above, the *Glossina* SGHV genome is a circular dsDNA molecule of 190,032 bp, with the putative ORFs distributed equally on both strands (51% forward, 49% reverse) and a gene density of one ORF per 1.2 kb (Abd-Alla *et al.*, 2008). Position one of the virus genome was arbitrarily assigned to the A of the ATG translational initiation codon of the ORF encoding a homolog of the baculovirus ODV p74 envelope protein. Many of the ORFs are clustered into inferred cassettes in both strands, and represent 86% of the genome. The genome has a high A+T content (72%). About 3% of the viral genome is composed of 15 repeat sequences distributed throughout the genome. As reported earlier (Odindo *et al.*, 1986), when *Glossina* SGHV was identified, the virus could not be assigned to any of the families of DNA viruses described at that time (Abd-Alla *et al.*, 2008). This was in consideration of its signature characteristics, i.e. the induction of SGH symptoms, possession of an enveloped rod-shaped viral particle, a large circular dsDNA genome, and being non-occluded (Abd-Alla *et al.*, 2010a). Based on these characteristics, the virus was proposed to be accommodated in a new virus family, *Hytrosaviridae*, a name derived from "*Hy*per*tro*phia *s*ialo*a*denitis", a Greek word for "salivary gland inflammation". The *Glossina* SGHV is commonly referred to as the salivary gland hypertrophy virus (GpSGHV), and is classified in the newly established *Hytrosaviridae* family, genus *Glossinavirus*, and species *Glossina hytrosavirus* (Abd-Alla *et al.*, 2009b). This taxonomy is now accepted by the ICTV (http://ictvonline.org/).

## Phylogeny of hytrosaviruses

Phylogenetic analysis of SGHVs based on the DNA polymerase gene, which is present in all large dsDNA viruses, does not cluster these hytrosaviruses with other insect dsDNA viruses (Abd-Alla *et al.*, 2008; Garcia-Maruniak *et al.*, 2009). Instead, the DNA polymerase of SGHVs clusters more closely to that of herpesviruses and other viruses with linear dsDNA genomes. Alignment-free method using whole proteome

phylogenetic analyses of dsDNA viruses shows close association of the SGHVs and nimaviruses (specifically the white spot syndrome virus; WSSV) (Gao and Luo, 2012; Wu *et al.*, 2009; Yu *et al.*, 2010). Despite the apparent ambiguities, these and other phylogenetic methods, such as super-tree and super-matrix methods (Wang and Jehle, 2009; Wang *et al.*, 2011), support the notion of a common ancestry of SGHVs with baculoviruses, nudiviruses and nimaviruses (Jehle *et al.*, 2013; Wang *et al.*, 2011). As mentioned above, SGHVs have been exclusively confirmed in dipteran species: *G. pallidipes*, *M. domestica*, and possibly *M. equestris*. It has been proposed that GpSGHV and MdSGHV are phylogenetically related to baculoviruses, but have evolved in a very close association with their respective dipteran hosts. The hytrosaviruses share 12 out of the 31 baculovirus core genes identified to date (Jehle *et al.*, 2013), and are therefore more distantly related to baculoviruses than for instance the nudiviruses: Nudiviruses share 20 of baculovirus core genes (Wang *et al.*, 2011). Nevertheless, these arguments appear to suggest a common ancestry of SGHVs, nudiviruses, baculoviruses, and possibly nimaviruses. These are all large circular, nuclear-replicating, invertebrate viruses with dsDNA genomes, and possibly form a virus superfamily.

## Rationale and scope of this thesis

The goal of this dissertation was to study the infectomics (defined here as the functional and structural genomics and proteomics) of GpSGHV. It was conceptualized that the data obtained from these studies would be useful to develop novel, rationally designed strategies to manage GpSGHV infections in the laboratory colonies of *G. pallidipes*. **Chapter 2** describes the dynamics and impacts of GpSGHV infection on the productivity of *G. pallidipes* colonies and the modes of the virus transmission. In **Chapter 3**, GpSGHV strains circulating in wild populations of *G. pallidipes* are investigated. **Chapter 4** investigates GpSGHV proteome, and correlates the viral ultra-structure to the protein composition, morphogenesis and cytopathology of the virus. In **Chapter 5**, the role of tsetse saliva in the transmission of GpSGHV is investigated by determining the secretome of asymptomatic and symptomatic *G. pallidipes*. **Chapter 6** investigates the interplay between GpSGHV and the tsetse microbiome in trans-generational virus transmission. **Chapter 7** describes an essential advancement in the management of GpSGHV in *G. pallidipes* colonies by modification of the *in vitro* membrane-feeding regime traditionally used in tsetse mass – production facilities. Finally, **Chapter 8** provides a synopsis on the extent to which various research goals described in this thesis were achieved, and proposed hypotheses in an attempt to explain some of the signature features of GpSGHV pathobiology.

# Chapter 2

# Dynamics of GpSGHV transmission in laboratory colonies of *G. pallidipes*[1]


## Abstract

Tsetse flies (Diptera; Glossinidae) are naturally infected by the *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV). GpSGHV infection can either be asymptomatic or symptomatic, with the former being the most rampant in these colonies. The asymptomatic state can be triggered to a symptomatic state, leading to detectable salivary gland hypertrophy syndrome (SGH). High prevalence of SGH symptoms has been associated with tsetse flies' reproductive dysfunction, which can lead to colony collapse of some tsetse fly species such as *G. pallidipes*. To gain a better understanding of the impact of GpSGHV in *G. pallidipes* colonies, and to follow development of SGH in the $F_1$ progeny of symptomatic flies, tsetse flies reared under different conditions were examined. The results demonstrated that, whereas the $F_1$ fly progeny of asymptomatic parents did not develop SGH, the $F_1$ fly progeny of symptomatic females mated with asymptomatic males had a high SGH prevalence (65 % in males and 100 % in females), and that these flies are sterile. Stress in the form of high fly densities in holding cages (≥ 100 flies per cage), and high temperatures (30°C) in the insectary led to high fly mortalities and low productivity. The numbers of viral particles secreted via saliva into blood during membrane feeding correlated with the infection statuses of the flies. After a single blood-feeding event, asymptomatic and symptomatic flies release an average of $10^2$ and $10^7$ viral genome copies per fly, respectively. Feeding of the flies on fresh blood meals at every feed for three fly generations significantly reduced the viral titres in these flies when compared with the viral titres in flies maintained under traditional feeding regime. The results of these studies allowed the initiation of colony management protocols aimed at minimizing the risk of horizontal GpSGHV transmission and enable establishment of SGH - free colonies.



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# Introduction

Tsetse flies (*Glossina* spp.) are the only vectors of a debilitating zoonotic disease in sub-Saharan Africa, sleeping sickness in humans and Nagana in cattle (Steelman, 1976). In many parts of sub-Saharan Africa, trypanosomoses and the presence of tsetse are considered as major obstacles to the development of sustainable livestock production systems and important 'root causes of hunger and poverty' (Dyck *et al.*, 2005; Feldmann *et al.*, 2005; Jordan, 1986). Due to the lack of effective vaccines and inexpensive drugs for sleeping sickness and the development of resistance of Nagana against available trypanocidal drugs (Aksoy and Rio, 2005), it is now generally accepted that control of the tsetse vector is the most efficient and sustainable management for trypanosomoses (Holmes and Torr, 1988; Leak, 1998; Schofield and Kabayo, 2008). The use of the sterile insect technique (SIT) as a component of an area wide integrated pest management (AW - IPM) approach (Klassen and Curtis, 2005) is a powerful tsetse fly control method as amply demonstrated by eradication of *Glossina austeni* from the Island of Unguja, Zanzibar (Vreysen *et al.*, 2000). Efficient implementation of SIT depends on successful maintenance of laboratory tsetse flies colonies to produce high quality males capable of competing with wild males for inseminating wild tsetse females (Hendrichs *et al.*, 2005).

In the laboratory colonies of the tsetse fly *Glossina pallidipes*, infection by the salivary gland hypertrophy virus (GpSGHV) can be either asymptomatic or symptomatic. The salivary gland hypertrophy syndrome (SGH), which can lead to reproductive dysfunction and sometimes collapse of some tsetse fly species, notably the *G. pallidipes* (Abd-Alla et al., 2007a) characterizes the symptomatic infection. Incidence of asymptomatic infections can be high in both field and colonized tsetse fly populations (Odindo, 1982). Asymptomatic infections are likely maintained through vertical transmission, either via milk gland secretions or through gonadal tissues. Low GpSGHV titre in these asymptomatic flies does not cause measureable impacts on host's fitness. On the other hand, symptomatic infection is associated with testicular degeneration and ovarian abnormalities (Jura *et al.*, 1988; Kokwaro *et al.*, 1990; Sang *et al.*, 1998; 1999), and affects the development, survival, fertility and fecundity of naturally- or experimentally-infected flies (Jura *et al.*, 1993; Sang *et al.*, 1997). In the natural (wild) tsetse fly populations, the incidence of symptomatic infections is low (zero – 5 %); majority of these flies harbour high levels of asymptomatic infections. Dissections of laboratory-bred *G. pallidipes* flies have revealed that up to 85% of these flies exhibit detectable SGH symptoms.

In nature, vertical (mother-to-offspring) GpSGHV transmission, either trans-ovum or through infected milk gland secretions, is thought to be the most likely mode of virus transmission (Jura *et al*., 1989; Sang *et al.*, 1986, 1998). On the other hand, in the laboratory-bred flies, horizontal (fly-to-fly) GpSGHV transmission during the *in vitro*

membrane feeding has been suspected to be a major contributor of the virus transmission because up to 10 cages with flies may be fed on the same membrane in succession. To have a better understanding of the dynamics of GpSGHV transmission under laboratory conditions, a simple and reliable quantitative polymerase chain reaction (qPCR) has been developed (Abd-Alla *et al.*, 2009a).

This chapter presents data on the investigations into the dynamics GpSGHV transmission in the laboratory colonies of *G. pallidipes.* Data is also presented on impact of stress (high temperature and high population density) on the prevalence of SGH, and the relationship between SGH and the colony productivity. Release of GpSGHV particles via saliva into blood meals during *in vitro* feeding is quantified and correlated with the occurrence of SGH symptoms. Some data are also presented on the conditions under which GpSGHV-infected individuals may convert from asymptomatic to symptomatic infection states.

# Materials and methods

## *Tsetse flies*

Two *G. pallidipes* colonies were used in this study. The first colony originated from pupae collected near Tororo, Uganda in 1975, colonized initially at the University of Leiden, The Netherlands, and subsequently transferred to the Insect Pest Control Laboratory (IPCL), Seibersdorf, Austria in 1982 (Tororo colony) (Feldmann, 1994a; Gooding *et al.*, 1997). The second colony was established at the Tsetse Fly Rearing and Irradiation Centre, Kality, Addis Ababa, Ethiopia from pupae collected near Arba Minch in the period 1999 - 2001 (Arba Minch colony). Unless otherwise stated, experimental flies were fed on heated, defibrinated bovine blood for 10-15 min, three times per week using an *in vitro* membrane-feeding technique, a method originally described by Langley and Maly (1969).

## *Feeding protocols*

Two feeding protocols were used. The first feeding protocol was the conventional or standard *in vitro* membrane feeding protocol, which is routinely used in tsetse mass–production facilities (Feldmann, 1994a). In this feeding method, up to ten successive cages of flies were offered a blood meal on the same membrane. The second feeding protocol (also based on the *in vitro* membrane feeding described above) was the "clean blood feeding protocol" (hereafter denoted as "clean feeding"), in which each cage of flies was provided with a fresh blood meal at each feeding event. Pupae produced from sequential larviposition events were collected and incubated at 24 °C until emergence.

## *Diagnosis and quantification of GpSGHV titres in live tsetse flies*

To detect GpSGHV-infected flies without dissection, a non-destructive polymerase chain reaction (PCR) method was used essentially as described by Abd-Alla *et al.*, (2007a). Briefly, total DNA was extracted from one mesothoracic leg excised from teneral (newly - eclosed, unfed) flies collected within 24 h post emergence, using ZR DNA genomic kit (Zymo Research, California, USA) according to supplier's instructions. DNA was eluted in 25-µl elution buffer and stored at -20 °C until further analyses. For PCR amplifications, 1.5 µl of the purified DNA was used as template. The PCR reactions were performed to amplify a 401 – bp fragment of the coding sequence of GpSGHV ORF5 (*odv–e66* gene; GenBank accession No. EF568108; Abd-Alla *et al.*, 2008). The following primers were used: GpSGHVfwd (5 – GCT TCA GCA TAT TAT TCC GAA CAT AC - 3), and GpSGHVrev (5 – GAT CCT GCT CGC GTA AAC CA - 3) (Abd-Alla *et al.*, 2007a). It should be noted that the *odv-e66* gene was selected for the analyses because it has no detectable polymorphism (Abd-Alla *et al.*, 2008). The PCR amplification products were analysed on a 1.5 % agarose gel. The PCR detection method is hereafter referred to as end-point PCR to distinguish it from the quantitative PCR (qPCR). The protocol that was used to quantify the viral genome copies in the extracted DNA (for whole flies) is described later.

## *Effect of stress on the prevalence of SGH symptoms*

Male and female teneral flies were randomly selected from the Tororo colony, and maintained in standard colony holding cages (20 cm diameter x 5 cm height) at different fly densities (38 to 180 flies per cage) and mating ratios (1:1 and 1:4, male: female) (**Table 1**).

**Table 1**: **Set up of the assay to determine effects of stress on SGH prevalence:** Seven treatments, each replicated at least three times were set up at different fly densities (30 – 180 flies per cage), and at different male (♂): female (♀) ratios to assess the effects of stress on SGH prevalence in *G. pallidipes* colonies.

| Treatment | No. of flies/cage | No. of ♂/cage | No. of ♀/cage | Ratio (♂:♀) |
|---|---|---|---|---|
| 1 | 38 | 8 | 30 | 1:4 |
| 2 | 75 | 15 | 60 | 1:4 |
| 3 | 112 | 22 | 90 | 1:4 |
| 4 | 150 | 30 | 120 | 1:4 |
| 5 | 60 | 30 | 30 | 1:1 |
| 6 | 120 | 60 | 60 | 1:1 |
| 7 | 180 | 90 | 90 | 1:1 |

In regular tsetse colonies, the normal fly density for the standard fly cages is 75 flies (at a male: female ratio of 1:4). All the treatments were replicated at least three times. The replicate cages for each treatment were maintained at temperatures of 24 °C or

30 °C for 21 days (equivalent to 7 blood meals or 3 times per week). After 21 days, all experimental flies were dissected to estimate occurrence of SGH symptoms.

### *Relationship between fly productivity and SGH prevalence*

To investigate the relationship between the prevalence of SGH symptoms and the productivity of experimental flies, 400 teneral virgin males and females were randomly selected from the Tororo colony, and separately maintained on clean blood meals until they reached sexual maturity. It is important to note that for the laboratory – bred *G. pallidipes* flies, maximum female receptivity occurs at 7 – 9 days of age, and willingness of males to mate is maximal at 12 – 14 days of age (Leegwater-van der Linden, 1981). It was not expected that the difference in sexual maturity between males and females could negatively affect the experimental results: the first egg is usually retained and remains viable for several days after female maturation, and would therefore permit ovulation in response to the mating stimulus (Wall, 1989).

To compose parental ($G_0$) generations, single matings of 10-day - old females and 7 day - old males were performed in individual plastic tubes (3 cm diameter and 6 cm height) with netting on top and bottom for feeding and pupae collection). Each tube containing the experimental flies was numbered to identify the individual mating partners. Flies were allowed to mate for 24 h, after which the males were removed and dissected to assess occurrence of SGH. Females were offered clean blood meals until they produced three $F_1$ larviposition pupae, or until 40 days post mating, whichever was the earlier. Subsequently, the female flies were also dissected to assess SGH. The $F_1$ pupae from individual females were collected in plastic tubes and labelled as described above for the parents.

Based on the parental SGH status, the $F_1$ pupae were divided into four groups, those from (i) asymptomatic male and female parents, (ii) asymptomatic male and symptomatic female parents, (iii) symptomatic male and asymptomatic female parents, and (iv) symptomatic male and female parents. Pupae from these four groups were incubated at 24 °C for 35 days or until emergence. Individual pair matings were made between $F_1$ males and females within each group of pupae and the males and females were treated as for the $G_0$. The $F_2$ pupae were collected from each individual female fly and incubated at 24 °C until emergence, or for 35 days. For the $F_1$ and $F_2$, the pupae that did not emerge by the $35^{th}$ day of incubation were considered dead.

### *Detection and quantification of GpSGHV in blood meals after feeding*

Symptomatic and asymptomatic tsetse flies (eight flies in each category; replicated three times) were fed individually on ~ 200 µl clean blood for 10 - 15 min. Only flies that were fully engorged at the end of the feeding events were further analysed. After

feeding, the blood that remained under the feeding membranes was collected for subsequent DNA extraction. For negative control, ~ 200 µl of the clean blood was sampled prior to each feeding event. Total DNA was extracted from the collected blood using the DNeasy kit (Qiagen) following the supplier's instructions, and virus was detected by the end-point PCR protocol as described above. Viral titres in the samples were quantified by qPCR as described below.

### *Controlled feeding of tsetse on GpSGHV - contaminated blood and analysis of virus particles secreted via saliva*

Teneral *G. pallidipes* flies were screened by end-point PCR to determine GpSGHV infection status. Sixteen symptomatic flies were selected, maintained individually in numbered plastic tubes, and fed on ~ 500 µl clean blood meals. After feeding, the blood remaining under the membranes was collected by pipetting, thoroughly mixed and divided into two aliquots (~ 200 µl each). One of the aliquot was used for DNA extraction and used directly for qPCR analyses (Abd-Alla *et al*., 2009 a). The other was used to feed asymptomatic (PCR - negative) flies. The PCR - negative flies were divided into 4 groups (each composed of 16 flies), and given one, three, five or seven successive GpSGHV-contaminated blood meals. After receiving the respective number of blood meals, the first three fly groups were offered clean blood meals to bring the total to seven blood meals. Negative control flies received seven meals on clean blood. All fly groups were offered an additional eighth clean blood meal, and the blood residue after this final feed was analysed by end-point PCR to detect GpSGHV.

### *Impact of clean feeding on GpSGHV titres*

Parental ($G_0$) generation for the bioassay consisting of 15 male and 60 female teneral flies was randomly selected from the Tororo colony. The flies were maintained on clean feeding for sixty days, and the pupae were collected. To determine GpSGHV titres at the start of the assay, twenty-four $G_0$ flies (six males and eighteen females) were randomly sampled and stored at – 20 °C for qPCR analysis. After incubation at 24 °C, the flies emerging from the $F_1$ pupae were subsequently mated and maintained as above, and $F_2$ pupae were collected. The $F_1$ and $F_2$ adults were then sampled and stored at -20 °C for qPCR analysis. For control, six males and eighteen females (sixty days-old) were selected from the Tororo colony that was maintained on a normal feeding regime. Total DNA was extracted from individual flies from all the samples using DNeasy kit (Qiagen), and GpSGHV titres determined by qPCR. All experiments were replicated three times.

### *Determination of GpSGHV titres by qPCR*

For qPCR analyses of viral genome copies present in the DNA extracted from the residue blood meals, the excised legs and whole fly bodies (see above), equilibration

and calibration curves were made. Briefly, genomic DNA was extracted from a SGH-positive fly and amplified by end-point PCR as described above. PCR product was purified and quantified by Nanodrop spectrometry, and genomic equivalent calculated according to standard protocols using the *G. pallidipes* genome size reported by Abd-Alla *et al*., (2008; 2009 a). From the estimates, 10-fold serial dilutions of the DNA were made for each qPCR assay. Each dilution was processed in triplicate on the same 96-well PCR plate with the test DNA samples. Non-template controls (NTC) were included in each of the triplicate assays. The specificity of the qPCR amplifications was ascertained by assessing the melting temperatures.

### *Statistical analysis*

To compare means of GpSGHV titres and SGH prevalence rates between the experimental fly groups, statistical analysis was performed according to Sokal and Rohlf (1981). To find out actual significant differences between the treatments (groups), analysis of variance (ANOVA) was followed by Tukey's HSD (honestly significant difference) Test for unplanned ("a posteriori") comparisons of means and Student's *t* - test for regression coefficients. Pairs of proportions were compared using the likelihood - ratio (G) test.

## Results

This study was designed to investigate; (i) the influence of fly density and environmental (or insectary) conditions on the titres of GpSGHV and on the prevalence or occurrence of SGH symptoms, (ii) the interplay between SGH prevalence, fly mortality and productivity, and (iii) the dynamics of acquisition of GpSGHV particles released by infected flies via saliva during the conventional *in vitro* membrane feeding routinely used in tsetse fly mass production facilities (refer to materials and methods described above).

### *Diagnosis of GpSGHV in live tsetse*

The PCR products amplified from the genomic DNA extracted from single legs or whole fly bodies was obtained according to the protocol described above. According to the strength of the band obtained on the gels, GpSGHV infection status in the fly colony could be divided into three categories: negative, slightly positive and strongly positive (**Figure 1**). Dissections of the flies from each of these three categories showed that the PCR-negative and slightly positive flies did not exhibit any detectable SGH symptoms. On the other hand, in the strongly positive fly group, 85 % of the analysed flies had readily detectable (overt) SGH symptoms. It is unclear why the remaining 15% of the strongly positive flies had no detectable SGH symptoms. It is tempting to speculate that these flies could develop full SGH symptoms later in their lifetime.

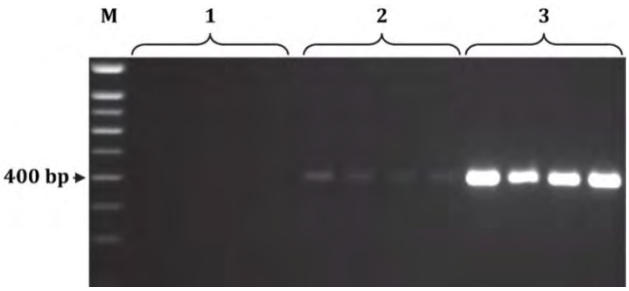


**Figure 1: End-point PCR diagnosis of GpSGHV infections in *Glossina pallidipes* flies:** The DNA was extracted from one mesothoracic leg from individual flies and amplified by end-point PCR. (1) Negative, (2) slightly positive and (3) strongly positive flies. M represent SF Smart DNA Ladder (Eurogentec).

## *Prevalence of GpSGHV in Seibersdorf and Kality G. pallidipes colonies*

The collapse of the two *G. pallidipes* colonies maintained at Seibersdorf in the past was associated with the high SGH prevalence. The first colony was initiated in 1982 using several batches of pupae collected from the Lambwe Valley, Kenya (*n*=528 pupae). The colony expanded slowly to reach 1,000 females in 1987, but low fecundity (1.1 pupae per female per month) and low insemination rates (< 85%) prevented further colony expansion. SGH was detected in 85% and 70% of males and females that were dissected. The second colony was established in Seibersdorf in 1999 from pupae collected near Arba Minch, Ethiopia, and reached 15,000 females in 2001. This colony steadily declined and became extinct in 2002. Dissections of flies from this colony revealed that overall, 85% of the flies had SGH (Abd-Alla *et al*., 2007a).

Between 2006 and 2009, regular dissection of batches of ~ 100 flies from the Tororo colony showed a stable SGH prevalence (less than 10 %). However, SGH prevalence in the Arba Minch colony maintained at the Tsetse Fly Rearing and Irradiation Centre, Kality, Addis Ababa, Ethiopia, showed significantly higher SGH rates of 22.4 % in 2007, 43.5 %, in 2008 and 27 % in 2009 **(Figure 2)** as compared to those obtained in the Tororo colony (10 % in 2007, $P<0.05$; 8 % in 2008, $P<<0.001$ and 10.33% in 2009, $P<0.01$). The data for 2007 and 2009 were obtained from flies dissected at Kality and the data for 2008 were obtained from flies dissected at IPCL, Seibersdorf, which had emerged from pupae originating from Kality. The high SGH prevalence has been accompanied by a decline in the size of the Kality colony.

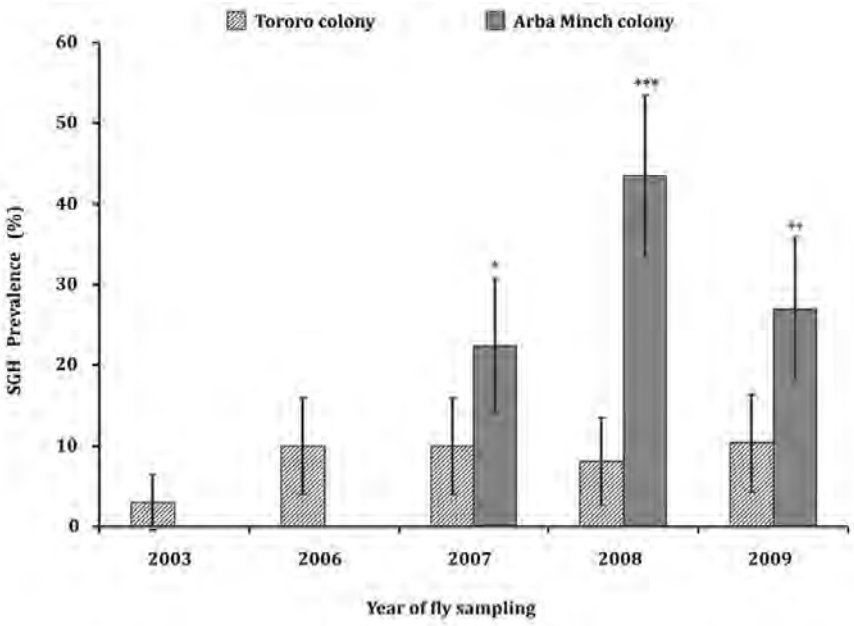


**Figure 2: Prevalence of SGH symptoms in *G. pallidipes* colonies:** The figure shows differences in the SGH prevalence in the Tororo *G. pallidipes* colony maintained at the IPCL, Seibersdorf, Austria, and the Arba Minch colony maintained at Kality, Addis Ababa, Ethiopia (likelihood ratio test, * $P<0.05$, ** $P<0.01$, *** $P<0.001$).

## *Effects of temperature and fly density on the prevalence of SGH*

Although testing GpSGHV-positive by a PCR (Abd-Alla *et al*., 2007a), a high proportion of *G. pallidipes* flies were asymptomatic. Under some unknown conditions, either environmental or genetic, asymptomatic individual can convert to symptomatic infection stats, the latter being characterized by detectable SGH symptoms. It is not clear how this happens. In other viral systems such as baculovirus, reactivation of asymptomatic/latent to symptomatic viral infection has been linked to factors such as overcrowding, temperature etc. (Hughes *et al*., 1993; see further discussions in **Chapter 8**). Potentially, similar factors may play roles in the appearance of SGH symptoms in the GpSGHV-infected *G. pallidipes.* As described above, an experiment was set up to test whether maintaining the experimental flies at high temperatures and high fly densities could have an effect on the expression of SGH symptoms in asymptomatic *G. pallidipes* flies. The results showed that, whereas at 24°C the number of flies per cage and the sex ratio had a limited effect on mortality, a significant increase in mortality was observed in flies reared at 30°C (**Figure 3**), especially at fly densities of >100 flies per cage regardless of the sex ratio.

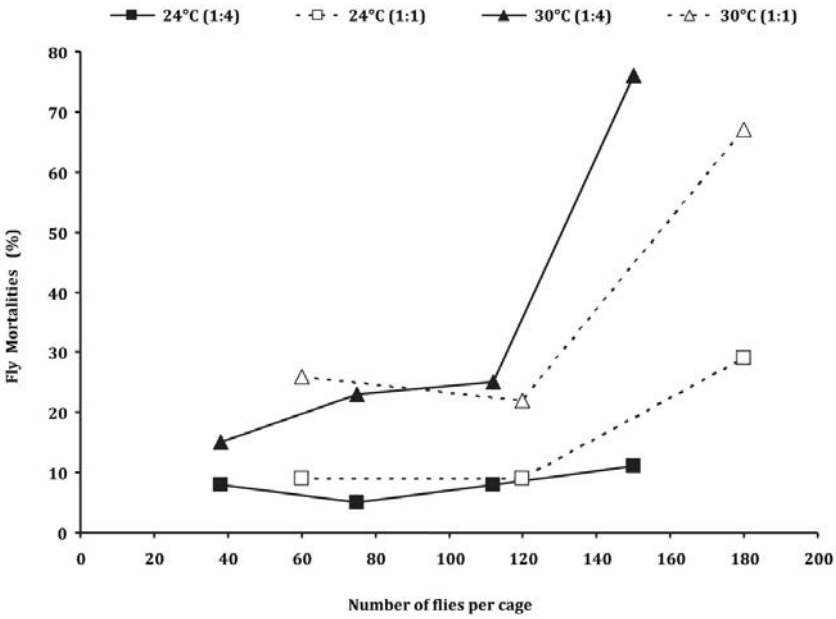


**Figure 3: Impacts of stress on fly mortalities:** The figure shows effects of fly density and temperature on mortality on flies sampled from the Tororo *G. pallidipes* colony (1:4, cages with sex ratio 1 male:4 female flies; 1:1, cages with sex ratio 1 male:1 female fly) after incubation for 21 days.

In the experimental flies that were maintained at 24 °C, female productivity progressively decreased with an increase in fly density regardless of the sex ratio. The females' productivity was significantly reduced at 30 °C, with the lowest productivity at a density of 180 flies per cage (**Figure 4**). Although SGH prevalence varied from zero - 4.5 % depending on treatment, there was no clear correlation between fly density or rearing temperature and the percentage of flies exhibiting detectable SGH. However, it cannot be excluded that the dead flies included a higher proportion of flies with SGH and that the symptomatic flies showed lower fecundity than the asymptomatic flies (Jaenson, 1986).

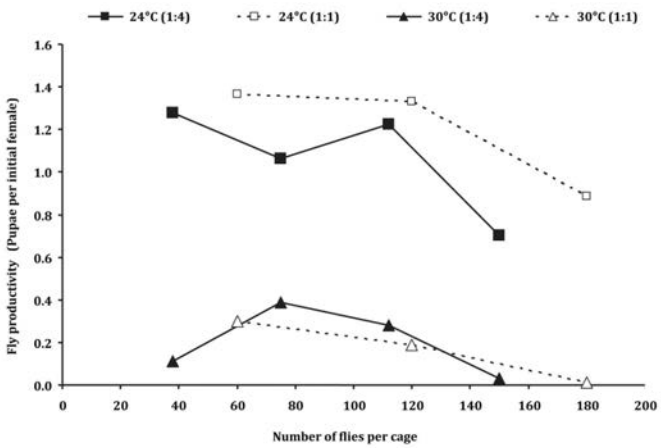


**Figure 4: Analysis of the impacts of stress on fly fecundity:** The figure shows the effects of fly density and temperature on pupae production from Tororo colony (1:4, cages with sex ratio 1 male:4 female flies; 1:1, cages with sex ratio 1 male:1 female fly).

## *Relationship between SGH and tsetse productivity*

Correlation of fly productivity with SGH is presented in **Table 2**.

**Table 2: Correlation between fly productivity and SGH:** Emergence rate, survival, mortality, productivity, and SGH prevalence of Tororo colony flies resulting from different mating combinations between symptomatic and asymptomatic flies.

| Generation | Sex | Categories | Fly groups | | | |
|---|---|---|---|---|---|---|
| | | | I (- ♀ x - ♂) | II (+ ♀ x - ♂) | III (- ♀ x + ♂) | IV (+ ♀ x + ♂) |
| $G_0$ | | No. of pairs | 21 | 16 | 16 | 1 |
| $F_1$ | Pupae | No. of pupae | 63 | 32 | 3 | 0 |
| | | No. of flies | 60 | 21 | 3 | 0 |
| | | % emergence | 95.24% | 65.62% | 100% | 0 |
| | Male | **No. of $F_1$ males** | 33 | 9 | 2 | 0 |
| | | No. dead before mating | 2 | 1 | 0 | 0 |
| | | % mortality | 3.03% | 11.11% | 0 | 0 |
| | | No. dissected (post mating) | 31 | 8 | 2 | 0 |
| | | No. with SGH | 0 | 5 | 1 | 0 |
| | | % SGH prevalence | 0 | 62.5% | 100% | 0 |
| | Female | **No. of $F_1$ females** | 27 | 12 | 1 | 0 |
| | | No. died | 3 | 5 | 0 | 0 |
| | | % mortality | 11.11% | 41.67% | 0 | 0 |
| | | No. dissected | 24 | 7 | 1 | 0 |
| | | No. with SGH | 0 | 7 | -* | 0 |
| | | % SGH prevalence | 0 | 100% | - | 0 |
| $F_2$ | | No. of $F_2$ pupae | 55 | 0 | 1 | 0 |
| | | Pupae per initial female | 2.03 | 0 | 1 | 0 |

*, The flies were dead before dissection; (-), asymptomatic fly;.(+), symptomatic fly

Dissection of 800 flies (400 males and 400 females) at the end of the $G_0$ showed an average SGH prevalence of 4.25 % in both sexes. From the 400 matings, 16 were classified in group II (negative males mated with positive females), 16 in group III (positive males mated with negative females), one in group IV (positive male mated with positive female), and the remainder (367) of the pairs were from group I (negative males mated with negative females) of which 21 pairs were selected randomly for further rearing and analyses (**Table 2**). The single pair in group IV did not produce any pupae. From the 16 pairs in group III, only two female flies produced three pupae, all of which emerged. Of these three $F_1$ pupae, two emerged as males, both of which had SGH; the $F_1$ female was asymptomatic and produced one $F_2$ pupa. All 16 females in group II produced an average of 1 to 3 pupae, totalling to 32 pupae. However, the pupae from this group had a reduced emergence rate (65%) and the emerged flies showed high mortality rates (11% in males, 42% in females). The mated $F_2$ females from this group did not produce any pupae, and subsequent dissections revealed that 62% of the $F_1$ males and 100% of the females (i.e. parents that produced the $F_2$ progeny flies) had SGH symptoms. Here, it should be noted that whereas the males were dissected earlier in the experimental period (i.e. after the 24-h mating period); the females were dissected at the end of the experimental periods (i.e. approximately 40 after mating).

These results confirmed previous observations that SGH affects the development, survival, fertility and fecundity of naturally infected (Jaenson, 1978, 1986; Sang *et al.,* 1997). In contrast to the group II flies described in the paragraph above, the 21 $G_0$ mated females of group I (both parents asymptomatic) produced 63 pupae that had an emergence rate of 95%. The $F_1$ flies were mated and dissection of the males showed that no individual had SGH symptoms. The $F_1$ females produced an average of two pupae per female and following dissection, no individual with SGH was found. In agreement with earlier studies (Jaenson, 1978, 1986), these results clearly indicated that males with SGH are usually sterile. The 2 out of 16 mating pairs with symptomatic males that produced at least one pupa could be explained by incomplete development of SGH symptoms in these males by the time of mating. Alternatively, the GpSGHV infection had not yet reached the reproductive organs.

## *Release of GpSGHV particles via saliva during feeding*

The levels of GpSGHV contamination in the blood used to feed asymptomatic and symptomatic flies were assessed by detection of viral DNA in the blood meals after a single feeding event. No viral DNA could be detected by the end-point PCR either in control blood, which was sampled before feeding events, or in the blood remaining after a single feeding event of asymptomatic flies (**Figure 6**). In contrast, viral DNA was easily detectable in ~50 % of the blood remaining after feeding symptomatic flies.

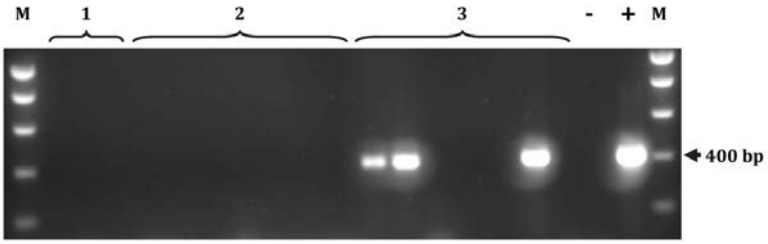


**Figure 6: Release of GpSGHV via saliva into blood meals during membrane feeding:** Lanes under numbers 1, 2, and 3 were loaded with PCR products of DNA extracted from blood before feeding, blood after feeding of asymptomatic and symptomatic flies, respectively. (-): negative control (non-template) and (+): positive control using DNA template extracted from purified virus, M: Smart DNA ladder (Eurogentec).

The lack of negative detectable GpSGHV DNA in the saliva secreted during feeding of almost 50% of the symptomatic flies in figure 6 was a surprising: one would expect that all symptomatic flies would release detectable viral genome copies during feeding, even by the end-point PCR. In the house fly virus, *Musca domestica* SGHV, the insects release a relatively steady amount of virus via saliva secretions per every feeding event between days 5 and 21 post infection (Lietze *et al*., 2009). To quantify the viral genome copies secreted via saliva into the blood during the feeding events of after feeding the asymptomatic and symptomatic flies, qPCR was performed using an optimized protocol described by Abd-Alla *et al*., (2009a). The results from qPCR clearly demonstrated that asymptomatic flies also released the virus into blood via saliva ($P < 0.01$) **(Figure 7)**.

The unsteady release of the virus into the blood via salivary secretion during feeding was unexpected. One would therefore question the qPCR quantification. However, the standard curve obtained in the qPCR (see **Figure 8**). The standard curve exhibited linearity ($R^2$) was close to one ($R^2$=0.997) over the whole range of the serial dilutions that were used (see **Figure 8 B**). The linear standard curve implied that the efficiency of the amplification was consistent at varying dilution concentrations. The number of PCR cycles that generated the linear standard fit slope fell within the acceptable parameters range of - 3.1 to - 3.6 (Slope = -3.248), implying the PCR amplification was sensitive. Further, the efficiency (E=98.9%) of standard curve fell within the standard acceptable parameters of 90%-110%. This implied that there were no contaminating DNAs (primers dimes, contaminating DNA, or PCR product) are present in the reaction as these would show up as additional peaks separate from the desired single homogeneous melt peak (**Figure 8 C**).

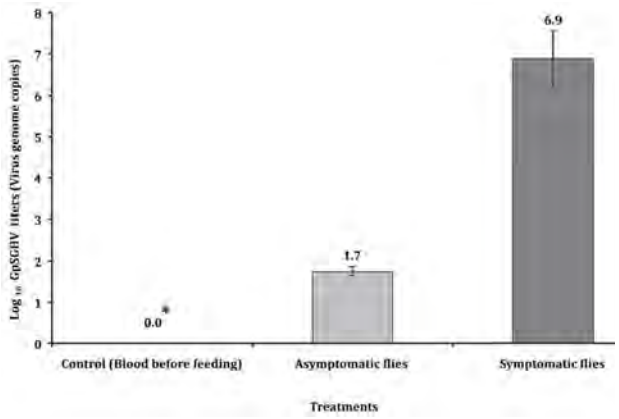


**Figure 7: Difference between release of virus via saliva into blood by asymptomatic and symptomatic flies during the *in vitro* membrane feeding:** The figure illustrates the virus titres ($log_{10}$) in blood before and after feeding asymptomatic and symptomatic Tororo *G. pallidipes* colony flies. (*: qPCR background value (0.35) in blood before feeding was subtracted from all values).

The presence of the single homogeneous melting peak far the dilutions confirmed specific amplification, and therefore the data for the reactions were deemed reliable and meaningful for further analysis and interpretation. The NTC sample, indicative of presence of primer dimers, showed a slight a small primer-dimer formation (see **Figure 8 A** and **B**), but this was acceptable because there was no corresponding peak in the amplification plots in the derived melting curve (see **Figure 8 C**); the threshold cycles (Cts) from the NTC wells were therefore trusted to be accurate.

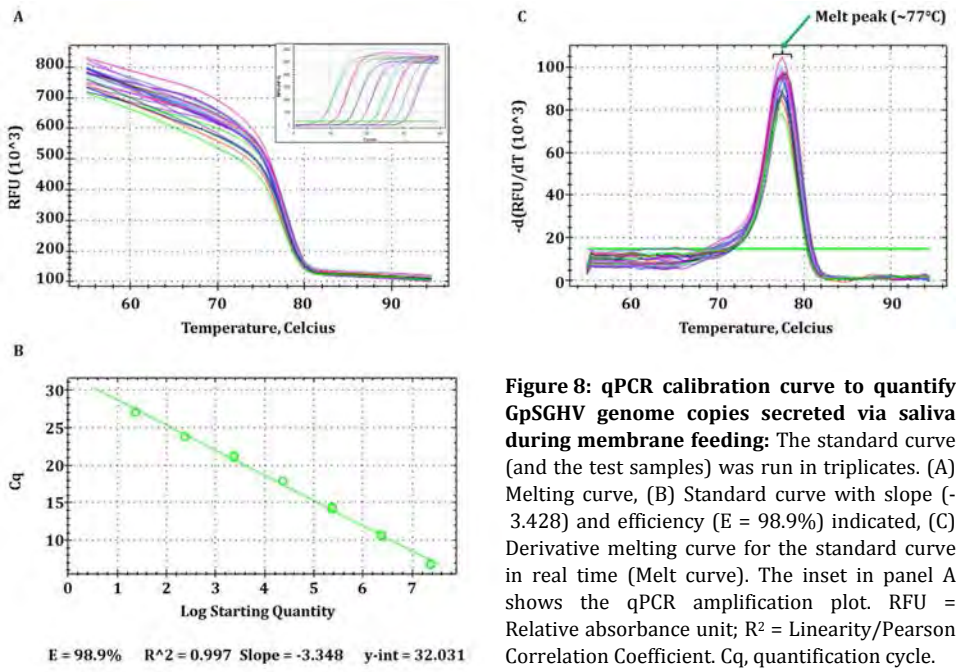


**Figure 8: qPCR calibration curve to quantify GpSGHV genome copies secreted via saliva during membrane feeding:** The standard curve (and the test samples) was run in triplicates. (A) Melting curve, (B) Standard curve with slope (-3.428) and efficiency (E = 98.9%) indicated, (C) Derivative melting curve for the standard curve in real time (Melt curve). The inset in panel A shows the qPCR amplification plot. RFU = Relative absorbance unit; $R^2$ = Linearity/Pearson Correlation Coefficient. Cq, quantification cycle.

## *Horizontal transmission of GpSGHV secreted via saliva*

The high numbers of viral genome copies found in the blood remaining under feeding membranes after feeding of the symptomatic flies, strongly supported, but did not confirm horizontal transmission of GpSGHV. To test the hypothesis that the released GpSGHV particles could be horizontally transmitted by feeding on virus - contaminated blood, the blood fed to symptomatic infected flies was re - fed to different groups of asymptomatic (PCR – negative) flies (16 flies each) for one, three, five and seven feeds (see materials and methods). At the end of the experimental period, flies were offered a final blood meal: the blood meals were collected and viral genome copies analysed by PCR. The results presented in **Figure 9** revealed that a single 10 – 15 min feeding on GpSGHV-contaminated blood meal was enough to detect the secretion of viral particles in almost 20 % of the previously PCR – negative flies. This proportion increased dramatically with the number of contaminated blood meals taken by the flies (regression coefficient = 11.975, t = 7.254, $P < 0.01$). QPCR was not conducted on salivary glands from these flies. It would have been interesting to quantify the viral genome copies present in the salivary glands in each of the groups of experimental flies.

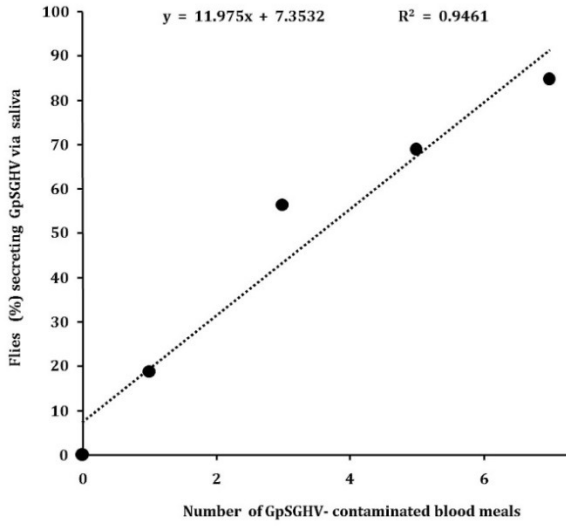


**Figure 9: Analysis of the horizontal GpSGHV transmission via fly saliva during membrane feeding of *G. pallidipes*:** The percentage of flies secreting viral particles into the blood during the feeding events in relation to the number of virus-contaminated blood meals received before. It should be noted that the flies secreting viral particles (shown as %) in the y-axis were determined from blood meals collected after the last (8th) blood meal that was offered to the flies after each respective number of GpSGHV-contaminated feeds, i.e. one, three, five and seven feeds (see text for details).

## *Impact of feeding clean blood on GpSGHV titres*

The observation that symptomatic flies released high numbers of GpSGHV particles into the blood during the membrane feeding raised the question of whether practicing clean feeding (as opposed to the conventional colony feeding described above), would reduce the viral titres in *G. pallidipes* colonies. A clear trend in reduction of average

GpSGHV titres in flies that were maintained on the clean feeding regime for three successive fly generations ($G_0$, $F_1$ and $F_2$), compared to the viral titres in flies maintained on the regular colony feeding regime ($P << 0.001$) (**Figure 10**). More importantly, there is a difference in $G_0$ and $F_2$.

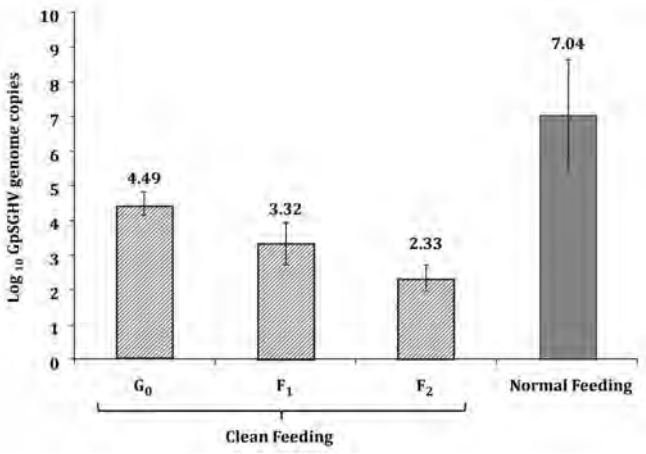


**Figure 10: Analysis of the effects of clean feeding on GpSGHV titres:** The figure shows the GpSGHV titres ($log_{10}$) in Tororo *G. pallidipes* colony flies maintained on the conventional feeding protocol and on clean feeding for three generations. It should be noted that the GpSGHV titres in the regular colony (shown in the normal feeding bar) were quantified in flies randomly selected from the colony and the viral titres are not equal to those in the $G_0$ flies, which were treated separately.

# Discussion

Collapse of two *G. pallidipes* colonies at the IPCL Seibersdorf was associated with a high SGH prevalence (Abd-Alla *et al.*, 2007a; Abd-Alla *et al.*, 2010b). After its establishment from pupae collected in Tororo, Uganda in 1982, the colony expanded slowly but stagnated in 1987 due to low fecundity (1.1 pupae per female per month) and low insemination rate (< 85 %). SGH was detected in 85 % of the male and 70 % of the female flies. The colony was re - established at Seibersdorf in 1999 from flies collected near Arba Minch, Ethiopia, and reached 15,000 female flies by 2001, but declined steadily, leading to its extinction in 2002.

The results presented in this chapter have demonstrated three categories of GpSGHV infections: (i) non - infected flies or flies presumed to be infected but with viral titres too low to be detected, (ii) flies with low viral titres and (iii) high viral titres (**Figure 1**). Using the qPCR method, GpSGHV infections could be classified into two broad categories: asymptomatic flies with low viral titres ($10^{4-7}$ viral genome copies), and symptomatic flies with high viral titres (estimated to be $10^{\geq 9}$ genome copies). These two categories corresponded to flies with few and abundant PCR products (by end – point PCR), respectively. Although GpSGHV infection (by PCR) in the Tororo colony was high (up to 100 %), fly dissections revealed that only 3.1 % of females and 3.8 % of males exhibited SGH symptoms. This is in contrast to the Arba Minch colony

where SGH prevalence was observed to be up to 43 % (unpublished data). It is yet to be demonstrated why there are high variations in the prevalence rates of asymptomatic infections in these two colonies. It should be noted that, experiences in handling of *G. pallidipes* colonies have revealed that the viral titres do fluctuate, the reason for which remains to be investigated.

The results presented in this chapter confirm previous observations that SGH affects the development, survival, fertility and fecundity of naturally or experimentally infected flies (Jaenson, 1978b; 1986; Sang *et al.*, 1997). The study also agrees with the earlier observations that males with SGH are usually sterile (Jaenson, 1978b; 1986). Only two out of sixteen pairing with symptomatic male flies produced only a single pupa. This could be explained by incomplete development of the SGH in the male at the time of mating or that the virus infection had not yet reached the reproductive organs (see **Table 1**). After demonstrating widespread asymptomatic virus infections in *G. pallidipes* colonies by end - point PCR, it was obvious that the reduction of fertility in the females and the cause of the sterility observed in the males could not be due to asymptomatic infections as the colony in Seibersdorf is stable and productive. This study revealed that sterility is linked to symptomatic infection, because symptomatic males are usually sterile when mated with asymptomatic females (group III matings in **Table 1**) or symptomatic females (group IV matings), while symptomatic females continue to produce progeny when mated with asymptomatic males.

Abd-Alla *et al*., (2009a) had previously reported that females with SGH produced pupae with high viral titres. However, it was not clear at that time whether or not these pupae would emerge, and whether any of the emerging flies would be fertile or not. The results from group II (see **Table 2**) clearly indicate that female flies with SGH produced a reduced number of pupae (about 50%), and that the $F_2$ female flies were completely sterile. The difference between the prevalence of SGH in the $F_1$ male flies and female flies produced by group II may be due to the males being dissected 24 h post mating whilst the females were maintained for 40 days before dissections, allowing more time for SGH symptoms to develop. No symptomatic flies were detected in the $F_1$ progeny from group I mating (asymptomatic males and females). These results demonstrated a clear correlation between the prevalence of SGH in both sexes and a reduction in colony productivity, which can explain the loss of the two colonies in 1987 and 2002 where 85% of individuals exhibited SGH symptoms. While no symptomatic flies were observed in the progeny of asymptomatic parents, the progeny of symptomatic females were all symptomatic and sterile. The symptomatic infected flies would therefore not be expected to produce offspring and the line would in this case die out from the colony in the same generation (in the case of the males) and in the next generation (in the case of the females). The data in this chapter shows that clean feeding leads to $\sim 60\%$ reduction in GpSGHV titres compared to the

conventional colony feeding regime (where up to 10 fly cages are fed on the same membrane in succession (See **Figure 10**). This is a huge impact on the fly progenies and is discussed further in **Chapter 7**.

Vertical transmission (mother to progeny) was not enough to explain the stable and increasing levels in symptomatic infected flies in the *G. pallidipes* colonies. It can be speculated that horizontal transmission of the virus occurred in laboratory colonies fed on the *in vitro* membrane system because contamination of the blood during the feeding has been reported (Abd-Alla *et al.*, 2007a). Possibly, the large variation in the GpSGHV titres detected in the blood after feeding symptomatic flies reflect variations for saliva released during each feeding event (see error bar in **Figure 7**). It should be noted that flies with heavily hypertrophied salivary glands are likely to have impaired production and release of saliva, which may explain the observed variations in the viral genome copy numbers (see **chapter 5**). Nevertheless, these results clearly show that symptomatic flies can release large numbers of viral particles into the blood during feeding, which is most probably a source of infection for the other flies in the fly colonies. These results are in agreement with those recently reported showing that a similar virus of the housefly, *Musca domestica* is transmitted *per os* (Lietze *et al.*, 2009). However, the release of GpSGHV particles into blood meals during membrane feeding is distinct from observations of the release of MdSGHV by the houseflies: Whereas tsetse flies seem to release variable amounts of viral particles, a relatively steady amount of MdSGHV was found to be orally released per housefly per feeding event (Lietze *et al*., 2009).

Although demonstrating that symptomatic flies contaminate blood during feeding, the question remained whether contaminated blood could be a source of infection for asymptomatic PCR-negative flies. The results confirmed that viral particles released into the blood during the feeding of symptomatic or asymptomatic flies' increases virus levels in previously PCR - negative flies, and that these flies subsequently start to secrete viral particles themselves. Surprisingly however, as shown in **Figure 6**, symptomatic flies did not secrete a constant amount of virus particles via saliva during feeding. Further, one would expect that the observed differences in the band intensities (see **Figure 6**) from saliva secreted by the symptomatic flies after feeding would be reflected in the qPCR results. This was not the case as shown in **Figure 7**. This apparent discrepancy between the end-point and qPCR results could shed a doubt on the reliability of the virus quantification. However, the standard curve used for this quantification (see **Figure 8**) indicated that these results were credible. The reason for these observations is unclear. However, there are indications that in *G. pallidipes,* the hyperplasia of the salivary glands is highly likely to impair the synthesis and release of saliva (Kariithi *et al*., 2011). Further, after a 10-15 min feeding event, results have shown that up to 20% and 30% of flies exhibiting SGH were either partially engorged or totally unfed, respectively (Kariithi, Unpublished

results). Eventually, approximately 20% of SGH-positive flies died of starvation or indigestion; the remaining SGH-positive flies hardly survive beyond 21 days post adult eclosion. These observations may explain the above-mentioned results. Again, as stated above, this situation is unlike the case of MdSGHV, whereby infected houseflies were found to secrete a constant of viral particles per feeding event (Lietze *et al*., 2009). It is not clear why the case of GpSGHV differs so markedly from that of MdSGHV. However, unlike in GpSGHV where expression of SGH symptoms is delayed, MdSGHV induces the onset of SGH at 3 dpi (See Figure 2 in Lietze *et al*., 2009). The results obtained from the assay on GpSGHV release via saliva may explain the high viral prevalence in laboratory-bred *G. pallidipes* maintained under the *in vitro* membrane-feeding regime where up to ten cages with flies are fed on the same membrane in succession. The absence of symptomatic flies in $F_1$ progeny from group I (asymptomatic parents) strongly support the hypothesis that feeding asymptomatic flies only on clean blood should progressively result in the disappearance of symptomatic infections from the colony.

## Concluding remarks

The following procedures were recommended for colony establishment and maintenance (See also Appendix I): (1) all flies introduced into a colony, whether from the field or another colony, should be pre - screened by PCR and any virus-infected individuals should be eliminated, (2) as far as practical, the number of cages with flies fed on the same membrane in succession should be reduced, (3) the colony should be routinely assessed for occurrence of SGH to enable corrective measures, and (4) as symptomatic individuals secrete numerous infectious virus particles, consideration should be given to manual screening and elimination of SGH-positive flies.

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# Chapter 3

# Prevalence and diversity of GpSGHV in wild *G. pallidipes* populations[2]


## Abstract

The salivary gland hytrosaviruses (SGHVs) are predominantly pathogenic to dipteran insects. This chapter presents results of investigations into the prevalence and genetic diversity of *Glossina pallidipes* SGHV (GpSGHV) in wild populations of the tsetse fly, *G. pallidipes* (Diptera; Glossinidae). While the majority of colonized *G. pallidipes* have asymptomatic infections, no information is available on the prevalence of asymptomatic infections in natural populations. In this chapter, GpSGHV diversity was examined using concatenated sequences of *p74*, *pif1*, *pif2*, *pif3* and *dnapol* viral genes. Polymerase chain reaction (PCR) analysis of 1,972 *G. pallidipes* samples collected from eleven geographical locations in six African countries revealed that 34% (*n*=672) of the samples were GpSGHV-positive. GpSGHV prevalence ranged from 2% to 100% from one location to another. Phylogenetic and gene genealogy analyses using the concatenated sequences of the five viral genes revealed low virus diversity. Whereas there was no correlation between GpSGHV diversity and geographical locations, virus haplotypes could be assigned to one of two distinct clades. The reference (Tororo) haplotype was the most widely distributed, and was shared by forty-seven individuals in seven of the eleven locations. Three Ethiopian haplotypes were restricted to one clade, and showed the highest divergence from the reference haplotype. This is the first report of molecular variability of GpSGHV in the natural populations of tsetse flies.



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# Introduction

Members of the family *Hytrosaviridae* are enveloped, rod-shaped, double-stranded DNA viruses that have been reported from three genera of Diptera (Abd-Alla *et al.*, 2009b; Abd-Alla *et al.*, 2010a). Hytrosaviruses replicate in the nuclei of salivary gland cells in adult insects, inducing salivary gland hypertrophy syndrome (SGH) with little obvious external symptoms (Abd-Alla *et al.*, 2010b; Lietze *et al.*, 2011a; 2012). Viral infection inhibits reproduction by suppressing vitellogenesis, causing testicular anomalies and disruption of mating behaviour of the dipteran host (Jaenson, 1978b; Jura and Davies-Cole, 1992; Lietze *et al.*, 2007; Mutika *et al.*, 2012; Sang *et al.*, 1998).

Due to the narrow host range of *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV), the geographical distribution of the virus is likely to be restricted to sub-Saharan Africa. In wild tsetse populations, SGH prevalence ranges from 0.08 to 15.6% (Burtt, 1945; Odindo, 1982). SGH occurs in all areas where tsetse flies have been sampled (Odindo, 1982; Otieno *et al.*, 1980). Factors such as ecosystems and tsetse fly density may influence the virus dynamics in wild populations of the insects (Challier, 1982; Odindo, 1982). In surveys of the incidence of SGH in *G. pallidipes* trapped in the field, Odindo (1982) reported monthly variations in viral infections from different sites in the Kenya. Some of the flies' sampling sites had consistently viral high infections throughout the sampling period regardless of the prevailing climatic factors. Odindo and Amutalla (1986) investigated the ecology of GpSGHV infection in *G. pallidipes* in forested ecosystems. The researchers reported a negative correlation between tsetse densities and viral infections: whereas tsetse fly densities were lowest in forested areas compared to fallow lands, the viral infections were highest in forested areas than in fallow lands. This observation led to the conclusion by the researchers that the low tsetse fly densities in forested areas might have resulted from virus-induced tsetse fly mortalities. Alternatively, healthy flies may have migrated to the fallow lands in search of food, thus leaving residue infected flies in the forested areas.

Attempts have also been made to identify potential animal reservoirs of *Glossina* hytrosavirus. Although GpSGHV has not been isolated from animal reservoirs, Odindo *et al.,* (1981) proposed that there might be a vertebrate host-tsetse-vertebrate host cycle in the dissemination of the virus. Field surveys for viral infections showed that young (newly emerged) flies exhibited SGH symptoms (Jaenson, 1978b; Odindo *et al.*, 1982), implying pre-eclosion viral infections. Since the virus is vertically transmitted (Jura *et al*., 1989, Sang *et al.*, 1996), variations in expression of SGH could be due to the differences in virus incubation periods and undefined factors that trigger asymptomatic flies to exhibit SGH. The fly's sex may also contribute to the dynamics of SGH prevalence. For instance, in the bulb fly, *Merodon equestris*, SGH is expressed higher in males (~ 88%) than in females (~16%) (Lyon, 1973). Similarly, in the house

fly, *Musca domestica* and in some wild tsetse species (*G. palpalis*, *G. pallicera*, and *G. nigrofucsa*), SGH prevalence has been reported to be two-fold (Geden *et al.*, 2008), and 5-fold (Gouteux, 1987), higher in males than in females, respectively. GpSGHV infections can lead to collapse of laboratory colonies of *G. pallidipes* (Abd-Alla *et al.*, 2007a; 2010b). The research described in this chapter was designed to assess the prevalence and diversity of GpSGHV in the natural *G. pallidipes* populations.

# Materials and Methods

## *Sample collection*

Adult *G. pallidipes* flies were sampled between 2005 and 2010 from ten geographical locations in eastern and southern African countries (**Figure 1**; **Table 3**) using acetone-baited traps (Dransfield *et al.*, 1991; Hargrove and Langley, 1990).

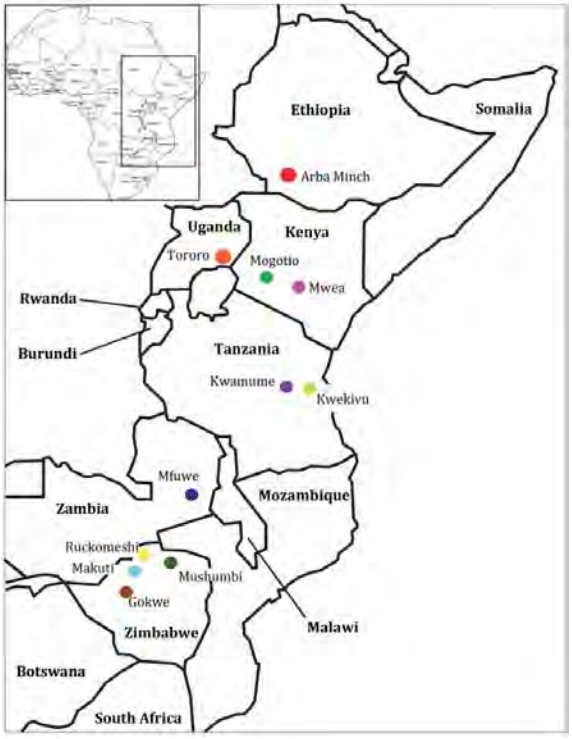


**Figure 1: Geographical locations of the sites where *Glossina pallidipes* flies were sampled for the study:** Adult *G. pallidipes* flies were sampled between 2005 and 2010 from ten geographical locations in eastern and southern African countries.

The collected samples were preserved in absolute ethanol, and shipped to the Insect Pest Control Laboratories (IPCL), Seibersdorf, Austria, for analyses. The *G. pallidipes* fly colony at the IPCL, originating from pupae collected near Tororo in Uganda in 1975, was used as the baseline (reference) isolate.

## *Determination of GpSGHV prevalence*

GpSGHV prevalence was determined by PCR using primers shown in **Table 1**.

**Table 1: Primers used to determine the prevalence and genetic diversity of GpSGHV:** Primer sequences used to amplify the *p74*, *pif1*, *pif2*, *pif3* and *dnapol* GpSGHV genes in prevalence and genetic diversity studies.

| Application | Target gene | Primer name | Primer sequence (5′- to -3′end) | Gene region | Size (bp) |
|---|---|---|---|---|---|
| Prevalence | *odv-e66* | ORF05-1F | GCATTCACAGCATCCCAATTTT | 4499-4520 | 401 |
| | | ORF05-1R | CTTGTCAGCGCCACGTACAT | 4880-4899 | |
| | | ORF05-2F | GATCCTGCTCGCGTAAACCA | 4421-4440 | 401 |
| | | ORF05-2R | GCTTCAGCATATTATTCCGAACATAC | 4796-4821 | |
| | *Dnapol* | ORF079F | GTACATATTCGAATGTATTTGCCGTTGCTC | 114624-114653 | 320 |
| | | ORF079R | CGGGAGGAGTTGTAATACCCTGTATCAAAG | 114915-114944 | |
| | **Degenerate primers** | | | | |
| | *p74* | p74-1F | TATMGWCCSGAGATWATGTCRCAC | 94-117 | 708 |
| | | p74-1R | MACRTTYGGATCRATAAARAAMGC | 826-849 | |
| | | p74-2F | TGTCARATWAATTATCCMCGYGGTAA | 232-257 | 373 |
| | | p74-2R | AARTCATCGCAATARTAYTTRTT | 583-605 | |
| | **Tsetse fly specific Primers** | | | | |
| | *GpCAG133* | GpCAG133F | ATTTTTGCGTCAACGTGA | | 180- |
| | | GpCAG133R | ATGAGGATGTTGTCCAGTTT | | 220 |
| Genetic diversity | *p74* | p74-Outer1F | ATTATGTGTCGGCCGATCGTTATGC | 26-50 | 689 |
| | | p74-Outer1R | CCCATCGAACACCATATTGAACAGCTTTA | 687-715 | |
| | | p74-Nes1F | GTATAGACCGGAGATTATGTCGCACG | 93-118 | 529 |
| | | p74-Nes1R | GGGCAAATTGCATTTTAAAGTCATCGC | 596-622 | |
| | | p74- Nes2F | CCGGAGATTATGTCGCACGTGTATGT | 100-125 | 439 |
| | | p74- Nes2R | CGCTCGTTAAAGTTAATATCAAACCCAGTACC | 508-539 | |
| | *pif1* | Pif1- Outer2F | CGATATAGTATTACCCAATCCGTG | 136001-136024 | 673 |
| | | Pif1- Outer2R | CAATGCCCCTATTGCCAAAGTTTGC | 136650-136674 | |
| | | Pif1- Nes1F | CAAGGGCATTGGAGAAGTAGTACTAG | 136055-136080 | 584 |
| | | Pif1- Nes2R | GTACCAGGAGGAGTTGGATACATGACGGAG | 136610-136639 | |
| | | Pif1- Nes2F | GGTAATAATGGGAAATACGCGAATGCAATGATGAG | 136158-136192 | 392 |
| | | Pif1- Nes2R | CACCACAGGTATAGTGCCATTGTAGACTCGTTTAC | 1365516-136550 | |
| | *pif2* | Pif2- Outer1F | CCAACTGAAACTCCGTGTCTAGAACCA | 65500-65526 | 753 |
| | | Pif2- Outer1R | CTTTGGGCAATTACCTCCAATTTCTACAACGACAA | 66218-66253 | |
| | | Pif2- Nes1F | CAATTACCTGATACCCAGATGTGC | 65546-65569 | 653 |
| | | Pif2- Nes1R | CCAATGGTTATTATTTAGGTCCAAATGAG | 66171-66199 | |
| | | Pif2- Nes2F | GATGGTTTAATGTATCCATCGCCAAC | 65611-65636 | 545 |
| | | Pif2- Nes2R | GGCAAAATGTAATATTAAATGTCAAGATGCGGATG | 66122-66156 | |
| | *pif3* | Pif3- Outer2F | GATGAATTGGATTTTACTGACCGTTG | 108318-108343 | 633 |
| | | Pif3- Outer2R | GCTCATTAAGTTGAAATCACCCCCGTCGTC | 108922-108951 | |
| | | Pif3- Nes1F | GATGGTTTCATAGTTATCTGAAAGAATTG | 108405-108433 | 484 |
| | | Pif3- Nes1R | CGAACGGTTTCAGTTTATCATCGTCAG | 108863-108889 | |
| | | Pif3- Nes2F | TCAAACGATTATCCGGACATTGAAC | 108523-108547 | 301 |
| | | Pif3- Nes2R | CTAGTCAATTCGTTTACCGCATAAC | 108800-108824 | |
| | *Dnapol* | DNApol-Outer2F | CGTCACGGAGAAAAGAAGCTCGATATTG | 114482-114509 | 616 |
| | | DNApol-Outer2R | CGAACTTCAGCACGGGATTGCAGCAAC | 115072-115098 | |
| | | DNApol-Nes1F | CACGATCGTGGTTCTAAAGAGGATTGC | 114533-114559 | 481 |
| | | DNApol-Nes1R | CGAGAAGTACCCGGCACGATGCGAC | 114990-115014 | |
| | | DNApol-Nes2F | GTACATATTCGAATGTATTTGCCGTTGCTC | 114624-114653 | 320 |
| | | DNApol-Nes2R | CGGGAGGAGTTGTAATACCCTGTATCAAAG | 114915-114944 | |

Total DNA was extracted from 1,972 individual (whole) fly bodies using either the DNeasy Tissue Kit (QIAGEN Inc., Valencia, CA), or the method described by Bender *et al*., (1983). Partial coding regions of *p74*, *odv-e66* and *dnapol* GpSGHV genes (GenBank accession number: EF568108) were amplified by PCRs using either GpSGHV-specific primers, or a set of degenerate p74 primers as previously described (Abd-Alla *et al.*, 2007a; 2008). These primers were used in single pairs or in multiplex PCR. The *G. pallidipes* microsatellite GpCAG133 gene sequence was used to control the quality of

the extracted DNA (Baker and Krafsur, 2001; Cheng and Aksoy, 1999). For all PCR amplifications, 22.5 µl of 1.1x pre-aliquoted PCR Master Mix (ABgene, UK) was used. In a final volume of 25 µl, the master mix contained 0.625 U Thermoprime Plus DNA polymerase, 75 mM Tris-HCl (pH 8.8), 20 mM $(NH4)_2SO_4$, 2.0 mM $MgCl_2$, 0.01% (v/v) Tween-20 and 0.2 mM each of the four dNTPs. To the master mix, 1.5 µl of the isolated DNA template, plus forward and reverse primers to a final concentration of 0.2 mM per primer was added. Samples were considered GpSGHV-infected if any of the expected viral PCR products were detected. Data were accepted only if the control gene (GpCAG133) sequence was successfully amplified.

## *Determination of GpSGHV genetic variation*

Genetic variation of GpSGHV in the field-caught *G. pallidipes* was assessed by PCR-amplifications of *p74, pif1, pif2, pif3* and *dnapol* genes. To overcome sequence heterogeneity at the primer binding sites and to amplify the same gene regions, three primer pairs flanking the targeted regions from each gene were designed (Kariithi *et al.*, 2013a). Annealing temperatures, primer combinations and specificity were optimized by gradient PCRs (37°C to 61°C) (**Table 2**), and by Nested PCRs according to standard protocols. PCR error rates were reduced by multiple independent PCRs.

**Table 2: PCR amplification conditions:** Primer combinations and annealing temperatures ($T_M$) used to amplify GpSGHV genes after optimization by gradient PCR (37°C to 61°C).

| Primer Combinations | Optimal $T_M$ (°C) | Target Gene | Amplicon size (bp) |
|---|---|---|---|
| P74- Nes2-F & R | 58 | *p74* | 439 |
| Pif1- Nes2-F & R | 60 | *pif1* | 392 |
| Pif2- Nes2-F & R | 60 | *pif2* | 545 |
| Pif3- Nes2-F & R | 58 | *pif3* | 301 |
| DNApol-Nes1-F & R | 58 | *dnapol* | 411 |

PCR products were purified by QIAquick PCR kit (Qiagen Valencia, CA), and sequenced by Sanger sequencing (MWG-BIOTECH AG, Germany). Only individual flies with good quality read for all the five genes were subjected to further analyses. Samples showing single nucleotides polymorphisms (SNP) or multiple peaks at any given nucleotide were sequenced from both ends with different independent PCRs.

## *Phylogenetic analysis and estimation of gene genealogies*

DNA sequence reads from the selected individuals were assembled using the SeqMan software (Lasergene 7.0, Dnastar Inc.) and the Open Reading Frame Finder platform (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The predicted ORFs were blasted against the NCBI non-redundant protein database (http://www.ncbi.nlm.nih.gov).

SNPs were determined using SeqMan and the selected gene sequences were used to assemble concatenated profiles resulting from the five sequenced viral ORFs.

## Analysis of GpSGHV phylogeny

The nucleotide and amino acid sequences of individual viral genes were used to determine phylogenetic relationships of the virus isolates/haplotypes. The corresponding genes of the *Musca domestica* SGHV (MdSGHV) were used as out-group. The sequences were aligned using a codon-guided version of the MAFFT method (Katoh *et al.*, 2002). Phylogenetic analyses were performed with PAUP version 4.0b10 (Swofford, 2002) using neighbour-joining (NJ), maximum likelihood (ML) and maximum-parsimony (MP) methods for a concatenated data set of the five partial protein-coding gene sequences. The PAUP programme was used to select the optimal evolution model by critically evaluating the selected parameters (Swofford and Sullivan, 2009), using the Akaike Information Criterion (Akaike, 1974). The approach suggested the GTR+G model (parameters: nucleotide frequencies of A = 0.32338; C = 0.18534; G = 0.20196; T = 0.28933; substitution rates of AC = 0.0014613, AG = 3.50963, AT = 1.18701, CG = 2.30311, CT = 3.56073, and GT = one; I (proportion of invariant sites) = none; and Γ (gamma distribution of among-site rate variation) = discrete approximation, with four rate categories). Under the selected model, the parameters and tree topology were optimized using the successive-approximations approach (Akaike, 1974; Sullivan *et al.*, 2005). The NJ and MP analyses (p distances) and ML analyses (heuristic search, random addition of sequences with 10 replicates, and tree bisection reconnection [TBR] branch swapping) were performed in PAUP. The robustness of nodes was assessed with 1,000 bootstrap replicates for NJ and MP and with 100 bootstrap replicates for ML.

## Analysis of GpSGHV gene genealogies

To determine the number of different GpSGHV haplotypes present in the wild *G. pallidipes* populations, the frequency of each haplotype in the population and the genetic distances within and between the viral haplotypes in these populations was assessed. A haplotype network approach was applied using the method of Templeton *et al.,* (1992; 1995): (here, haplotype is defined as a group of viral isolates possessing an identical set of alleles at separate loci in the sequenced genes). The maximum likelihood topology was translated into an un-rooted network, describing the optimal maximum likelihood topology under the maximum-parsimony criterion to map the number of mutations assigned to each branch of the network. Comparisons of the levels of genetic diversity within the haplotypes were performed using ARLEQUIN version 3.1 (Excoffier *et al.*, 1992; 2005). Briefly, the ten geographical locations, including the Tororo reference isolate (**Figure 1**) were used to define the structure of the virus haplotypes. The output file was loaded into the HapStar program, version 0.5

(Teacher and Griffiths, 2011). HapStar uses a spring model algorithm (Tollis *et al.*, 1998) by automatic repulsion of disconnected haplotype branch nodes while at the same time attracting the connected ones until they reach an optimal format. The GpSGHV haplotype network was generated by running several successive iterations until the branch lengths gradually relaxed into an optimal state. The optimal layout of the network was exported as a scalable vector graphics (SVG) file into Inkscape graphics editor software (http://inkscape.org/) for addition of colours and text.

# Results and Discussion

## *GpSGHV prevalence in wild tsetse populations*

The SGH prevalence in wild tsetse fly depends on the geographical location, seasonality, age and the distribution of tsetse species. For instance, Ellis and Maudlin (1987) found that 0.4% and 2.0% of wild-caught *G. m. morsitans* and *G. pallidipes* in Zambezi Valley, Zimbabwe, had SGH, respectively. Further, Gouteux (1987) reported that at onset of the rainy season, the prevalence of the SGH for females and males, respectively, was 0.31% and 0.26% in *G. palpalis*; 1% and 4.55% in *G. pallicera*; and 0% and 1.15% in *G. nigrofucsa*. Odindo (1982) found SGH prevalence ranging from 0.9% to 15.6%. Of these, 29.6% of SGH occurred in flies that were > 40 days old. The highest rate of infection (15.6%) was observed in flies collected in the cold season in only one of the trapping sites (Odindo, 1982), 4 km from an area which recorded only 2.7% infection. Otieno *et al.*, (1980) reported that the incidence of SGH varied from 0.9% in Meru National Park to 5.4% in the Simba Hills National Park Reserve in the Central and the Coast of Kenya, respectively. Similar to the study by Odindo, there was no incidence of SGH observed in *G. longipenis* from the Meru National Park.

Majority of GpSGHV infections do not cause detectable symptoms (Abd-Alla *et al.*, 2009b); suggesting that GpSGHV prevalence in the field is higher than previously reported (see **chapter 1**). PCR method is more sensitive than dissection, which relies on occurrence SGH and not on direct quantification of GpSGHV titres. In this study, GpSGHV prevalence was determined by PCR by testing 1,972 flies sampled various locations (**Figure 1**; **Table 3**). While all the collected fly samples from the ten geographical locations tested positive for the control gene sequence (GpCAG133), the GpSGHV infection prevalence based on the specific PCR detection averaged 34.08 % ($n$=672) of the collected flies and varied widely from 2% in Mashumbi (Zimbabwe) to 88% in Kwekivu (Tanzania). In four of the ten locations, the viral prevalence was more than 66%, whereas the virus prevalence in a large proportion of fly sample ($n$=797) did not exceed 10%. These apparent spatial (and perhaps temporal) variations in GpSGHV prevalence may reflect geographical differences in susceptibility of tsetse populations to the virus, movement of susceptible flies, genetic variability of the virus and/or host, and density of the susceptible host population.

**Table 3: Details of *G. pallidipes* sample collection sites in Africa:** samples were collected from different geographical sites in eastern and central African countries for the analysis of GSGHV prevalence and genetic diversity

| Country | Location | Latitude | Longitude | No. tested | Infected flies (%) | Year |
|---|---|---|---|---|---|---|
| Uganda* | Tororo | 0° 41' 34.0002" | 34° 10' 51.9954" | 12 | 12 (100) | 2010 |
| Ethiopia | Arba Minch | 6° 7' 0.012" | 37° 1' 59.9874" | 431 | 297 (68.9) | 2006 |
| Kenya | Mwea | 0° 53' 13.884" | 37° 37' 59.268" | 428 | 20 (4.6) | 2007/8 |
| | Mogotio | 0° 9' 56.844" | 36° 6' 20.3394" | 369 | 36 (9.7) | |
| Tanzania | Kwekivu | 5° 44' 54.312" | 37° 52' 55.488" | 50 | 44 (88) | 2005 |
| | Kwamume | 5° 44' 54.312" | 37° 22' 55.488" | 44 | 29 (66) | |
| Zambia | Mfuwe | 13° 7' 40.5114" | 31° 46' 33.7074" | 201 | 143 (71) | 2007 |
| Zimbabwe | Mashumbi | 16° 10' 12.9714" | 30° 34' 0.0114" | 50 | 1(2) | 2006 |
| | Gokwe | 18° 13' 0.012" | 28° 55' 59.988" | 193 | 47 (24.4) | |
| | Ruckomechi | 16° 7' 59.988" | 29° 23' 59.9994" | 98 | 38 (38.8) | |
| | Makuti | 16° 18' 0" | 29° 15' 0" | 96 | 9 (9.4) | |
| **Total** | | | | **1,972** | **672 (34.08)** | |

* = colony established in Seibersdorf since 1978 and used as the reference virus isolate.

This study demonstrate that GpSGHV prevalence in the sampled locations is significantly higher than the 0-15% prevalence of the SGH reported in earlier surveys (Jura *et al.*, 1988; Kokwaro *et al.*, 1990; Sang *et al.*, 1998; 1999). Whether GpSGHV is stably associated with tsetse fly populations at specific geographical sites remains to be determined, but its detection in the areas studied indicates that the virus is probably present in most *G. pallidipes* populations. In 431 of the tsetse fly samples collected in Arba Minch (Ethiopia), GpSGHV prevalence was higher in males (91%) than in females (46.7%). However, positive PCR signals do not *per se* imply symptomatic infections that influence negatively on fly fitness. The flies used in this study were not dissected to assess incidence of SGH: these flies were presumed to be asymptomatic.

## *Sequence comparison and analysis*

The lack of a suitable cell system precluded GpSGHV cloning. Therefore, the alleles detected for the five viral genes in a single fly were considered as a single virus isolate. Alignment of the concatenated sequences of the five viral genes showed 99.2 to 100% nucleotide identity to the reference Tororo genome sequence. Comparison of the sequence data revealed nucleotide substitution rates for the different genes ranging from 0.8% (*pif3*) to 3.1% (*pif2*)**.** Twenty-seven single-nucleotide polymorphisms (SNPs) were detected (~1.8% of analysed nucleotides), twenty-one (84.0%) of which

were synonymous or neutral. The remaining six SNPs (16.0%) were non-synonymous, causing 1.2% (6/496) amino acid mutations (**Table 4**).

**Table 4: Analysis of single nucleotide polymorphisms (SNPs) detected in *G. pallidipes*:** Summary of information for the nucleotide polymorphisms detected in the partial sequences of GpSGHV.

| Gene | Length (bp) | No. of SNP[1]/total no. of nucleotides (%) | No. of silent nucleotide substitutions/total no. of nucleotide substitutions (%) | No. of amino acid mutations (%) |
|---|---|---|---|---|
| *p74* | 320 | 5/320 (1.56) | 2/3 (33.33) | 3/106 (9.43) |
| *pif1* | 296 | 4/296 (1.35) | 3/4 (75) | 1/98 (1.02) |
| *pif2* | 418 | 13/418 (3.11) | 12/13 (92.3) | 1/139 (2.16) |
| *pif3* | 251 | 2/251 (0.8) | 2/2 (100) | 0/83 (0) |
| *dnapol* | 212 | 3/213 (1.41) | 2/3 (66.66) | 1/70 (1.42) |
| **Total** | **1,497** | **27/1498 (1.80)** | **21/25 (84.00)** | **6/496 (1.21)** |

Majority of nucleotide polymorphisms detected at specific loci were due to single base substitutions, with only one of the 27 loci having a double nucleotide substitution. Investigation into the prevalence and diversity of MdSGHV in wild populations of house fly revealed similar findings (Prompiboon *et al.*, 2010). In MdSGHV, only 11% of non-synonymous substitutions resulted in 28 amino acid substitutions: the remaining 89% were synonymous or neutral. Although the samples used for the MdSGHV analyses represent a much wider geographical area, variation among the MdSGHV populations was also noted to be low (Prompiboon *et al.*, 2010).

The sequence chromatograms and sequencing of independently acquired amplicons from both ends revealed in some flies multiple peaks for one or more loci. This observation may be speculated to imply the existence of co-infection with more than one GpSGHV genotype. The presence of multiple genotypes in single isolates can contribute to the maintenance of diversity of insect viruses (Clavijo *et al.*, 2010). It has been suggested that viral diversity within infected hosts may influence virulence, within-host population dynamics and virus fitness (Cicin-Sain *et al.*, 2005; Hodgson *et al.*, 2004; Simön *et al.*, 2006). It should be noted that the sequences having multiple peaks were not cloned, thus limiting the conclusions of the data presented here (see **Chapter 8** for further discussions).

## *Phylogeny and genealogical relationships of GpSGHV*

PCR analysis showed that 672 individual tsetse fly samples were GpSGHV-infected, of which 228 were sequenced for *p74*, *pif-1*, *pif-2*, *pif-3* and *dnapol* viral genes (**Table 5**). Of the 228, 136 individuals had good quality sequence reads for all the five viral genes. The remaining 92 samples gave truncated reads, which matched respective genes.

**Table 5: Alleles of GpSGHV genes in different locations of tested countries:** Numbers between brackets indicate the number of tested sequence for each allele.

| Country | Location | Tested flies | GpSGHV alleles (No of tested sequences) | | | | |
|---|---|---|---|---|---|---|---|
| | | | *p74* | *pif 1* | *pif 2* | *pif 3* | *dnapol* |
| Uganda* | Tororo | 12 | 1 (12) | 1 (12) | 1 (12) | 1 (12) | 1 (12) |
| Ethiopia | Arba Minch | 48 | 1 (41) | 1 (42) | 2 (42), 3 (6) | 2 (11), 3 (4) | 2 (10) |
| Kenya | Mogotio | 34 | 1 (15), 5 (10) | 1 (30), 3 (2), 4 (2) | 4 (32), 5 (2) | 1 (30). 3 (4) | 1 (28), 3 (2) |
| | Mwea | 19 | 1 (16) | 1 (18) | 1 (9), 4 (7), 7 (1) | 1 (18) | 1 (18) |
| Tanzania | Kwekivu | 11 | 2 (10) | 2 (11) | 4 (11) | 1 (11) | 1 (1), 3 (10) |
| | Kwamume | 7 | 1 (1), 2 (2), 4 (1) | 1 (6), 2 (1) | 1(5), 4 (1), 6 (1) | 1 (7) | 1 (3), 3 (4) |
| Zambia | Mfuwe | 52 | 1 (29), 3 (2) | 1 (51),4 (1) | 1 (40) | 1 (15) | 1 (10), 3 (6) |
| Zimbabwe | Mashumbi | 9 | 1(6), 4 (1), 6 (1) | 1 (9) | 1 (9) | 1 (9) | 1 (8) |
| | Gokwe | 21 | 1 (7), 4 (6) | 1 (17), 4 (1) | 1 (11), 3 (1) , 4 (1) | 1 (14) | 1 (14), 2 (1) |
| | Rukomeshi | 9 | 1 (1), 4 (5) | 1 (6) | 1 (8) | 1 (9) | 1 (7), 4 (1) |
| | Makuti | 16 | 1 (6), 4 (5) | 1 (11) | 1 (11) | 1 (16) | 1 (11) |
| **Total** | | **228** | **176** | **220** | **210** | **150** | **146** |
| **Alleles** | | | **6** | **4** | **7** | **3** | **4** |

* = colony established in Seibersdorf used as the reference isolate.

Further analysis resulted in 23 distinct profiles or haplotypes (**Table 6**). Concatenated sequences of these genes were aligned and analysed phylogenetically. The rationale of using the putative *per os* infectivity factors (PIFs) was mainly due to their strong homology to corresponding genes in baculoviruses and nudiviruses (Abd-Alla *et al.*, 2008). In some baculoviruses viruses, PIFs are critical in establishment of productive infection through the host insect mid-guts (Braunagel and Summers, 2007). It is presumed that GpSGHV PIF homologs play similar functions in hytrosaviruses. However, it should be noted that in the wild tsetse populations, GpSGHV is reported to be vertically transmitted (from mother to the developing offspring). Therefore, if the GpSGHV PIF homologs are involved in the initial virus infection, these proteins are likely to target the developing larval gut receptors. The implication of this is that PIFs may not be the ideal candidate genes to investigate GpSGHV genetic diversity.

The topology of the maximum likelihood phylogenetic tree illustrated that the diversity among the GpSGHV haplotypes is very low, and that there is very low resolution in the phylogeny. Two main clades were visible (Clades I and II; **Figure 2**), although these are rather poorly supported by the bootstrap values. Haplotypes from the same fly populations or geographical locations did not fall within any monophyletic groups in the topology. Except for few haplotypes, most of the haplotypes were randomly distributed with respect to geographical origin. Some of

the clustered haplotypes included haplotypes H2, H3 and H4 (Arba Minch), and H6 to H10 (Mogotio) (**Figure 3**).

**Table 6: GpSGHV haplotypes found in the same individuals collected African countries:** The frequency of the occurrence of the haplotypes is shown in the last column. The number in the parenthesis indicates the total number of flies in which the particular haplotype was detected.

| Country | Location | Flies tested | No of gene profiles | *p74* | *pif1* | *pif2* | *pif3* | *dnapol* | Haplotype No. | Frequency |
|---|---|---|---|---|---|---|---|---|---|---|
| Uganda* | Tororo | 12 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 12(12) |
| Ethiopia | Arba Minch | 14 | 3 | 1 | 1 | 2 | 2 | 2 | 2 | 6(14) |
| | | | | 1 | 1 | 2 | 3 | 2 | 3 | 4(14) |
| | | | | 1 | 1 | 3 | 2 | 2 | 4 | 4(14) |
| Kenya | Mogotio | 26 | 6 | 1 | 1 | 4 | 1 | 1 | 5 | 11(26) |
| | | | | 1 | 1 | 4 | 3 | 1 | 6 | 1(26) |
| | | | | 1 | 3 | 4 | 3 | 1 | 7 | 1(26) |
| | | | | 5 | 1 | 4 | 1 | 1 | 8 | 11(26) |
| | | | | 1 | 4 | 5 | 3 | 1 | 9 | 1(26) |
| | | | | 1 | 4 | 5 | 3 | 3 | 10 | 1(26) |
| | Mwea | 16 | 3 | 1 | 1 | 7 | 1 | 1 | 11 | 1(16) |
| | | | | 1 | 1 | 4 | 1 | 1 | 5 | 5(16) |
| | | | | 1 | 1 | 1 | 1 | 1 | 1 | 10(16) |
| Tanzania | Kwekivu | 10 | 1 | 2 | 2 | 4 | 1 | 3 | 12 | 10(10) |
| | Kwamume | 8 | 3 | 1 | 2 | 4 | 1 | 3 | 13 | 4(8) |
| | | | | 1 | 2 | 1 | 1 | 1 | 14 | 2(8) |
| | | | | 2 | 1 | 1 | 1 | 3 | 15 | 2(8) |
| Zambia | Mfuwe | 12 | 4 | 1 | 1 | 1 | 1 | 3 | 16 | 4(12) |
| | | | | 1 | 4 | 1 | 1 | 3 | 17 | 1(12) |
| | | | | 3 | 1 | 1 | 1 | 1 | 18 | 1(12) |
| | | | | 1 | 1 | 1 | 1 | 1 | 1 | 6(12) |
| Zimbabwe | Rukomeshi | 7 | 3 | 4 | 1 | 1 | 1 | 1 | 19 | 5(7) |
| | | | | 4 | 1 | 1 | 1 | 4 | 20 | 1(7) |
| | | | | 1 | 1 | 1 | 1 | 1 | 1 | 1(7) |
| | Mashumbi | 8 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 6(8) |
| | | | | 4 | 1 | 1 | 1 | 1 | 19 | 1(8) |
| | | | | 6 | 1 | 1 | 1 | 1 | 21 | 1(8) |
| | Gokwe | 12 | 4 | 1 | 1 | 1 | 1 | 1 | 1 | 6(12) |
| | | | | 4 | 1 | 1 | 1 | 1 | 19 | 4(12) |
| | | | | 4 | 4 | 4 | 1 | 1 | 22 | 1(12) |
| | | | | 4 | 1 | 3 | 1 | 1 | 23 | 1(12) |
| | Makuti | 11 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 6(11) |
| | | | | 4 | 1 | 1 | 1 | 1 | 19 | 5(11) |

* = colony established in Seibersdorf and used as the reference isolate.

The apparent complexity and diversity of GpSGHV haplotypes in the sampled locations (**Figure 3**) poses a question on the dynamics of the virus in wild tsetse populations. Potentially, over the years, the virus has undergone multiple rearrangement events to generate novel haplotypes. Since the main GpSGHV transmission route in natural tsetse fly populations is vertical i.e. mother-to-progeny transmission (Abd-Alla *et al.*, 2011b), this might explain the finding of several haplotypes in the same location, but it cannot explain how the mutations could arise independently in separate family lines within and between geographical locations. It is noteworthy that the phylogenetic out-group (MdSGHV) showed a divergence of several orders of magnitude from the GpSGHV haplotypes.

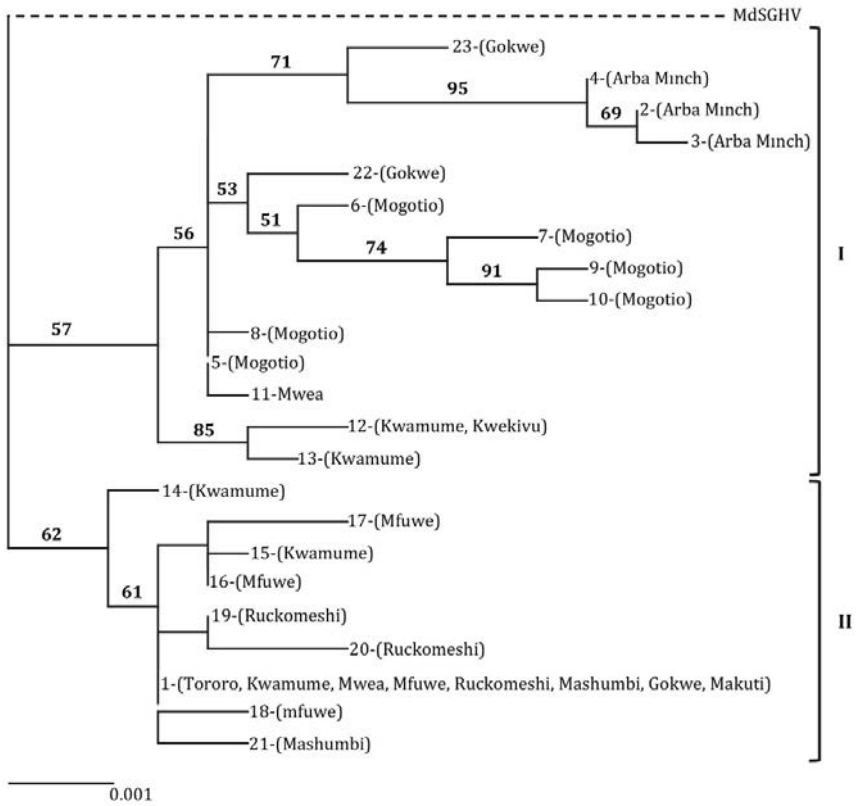


**Figure 2: GpSGHV haplotypes:** ML phylogenetic tree for all the 23 isolates of GpSGHV from 11 geographical locations in eastern and central African countries. ML bootstrap values based on 1,000 replicates are shown above the branches (only values larger than 50 are depicted). The bar at the lower left corner represents a branch length of 0.1% likelihood distance. The MdSGHV out-group branch (dotted line) has been shortened for improved visualization of GpSGHV relationship because it showed a divergence of several orders of magnitude from the GpSGHV haplotypes.

The frequency of the occurrence of GpSGHV infections (refer to **Table 5**) was used to examine the associations between the virus haplotypes by automated HapStar network program (**Figure 3**). The figure shows two star-like relationships of haplotypes, cantered on the two most abundant haplotypes (H1 and H5). Haplotype H1 has a wide distribution and was detected in seven out of 11 geographical locations (all except Ethiopia and Tanzania). Haplotype H19, which differs from H1 by a single mutation step, was present in four different populations, whereas haplotype H5, which differs from H1 by five single mutations, was found in two populations. All other haplotypes were restricted to a single population/geographic location. Compared to other geographical locations, more divergence was observed among the Kenyan haplotypes (**Figure 3**): nine haplotypes were detected in two locations; three

in Mwea (H1, H5 and the H11), and six in Mogotio (H5 to H10). One individual except for H5 and H1 represented these Kenyan haplotypes, which were represented by 11 and 10 individuals, respectively (**Table 6**).

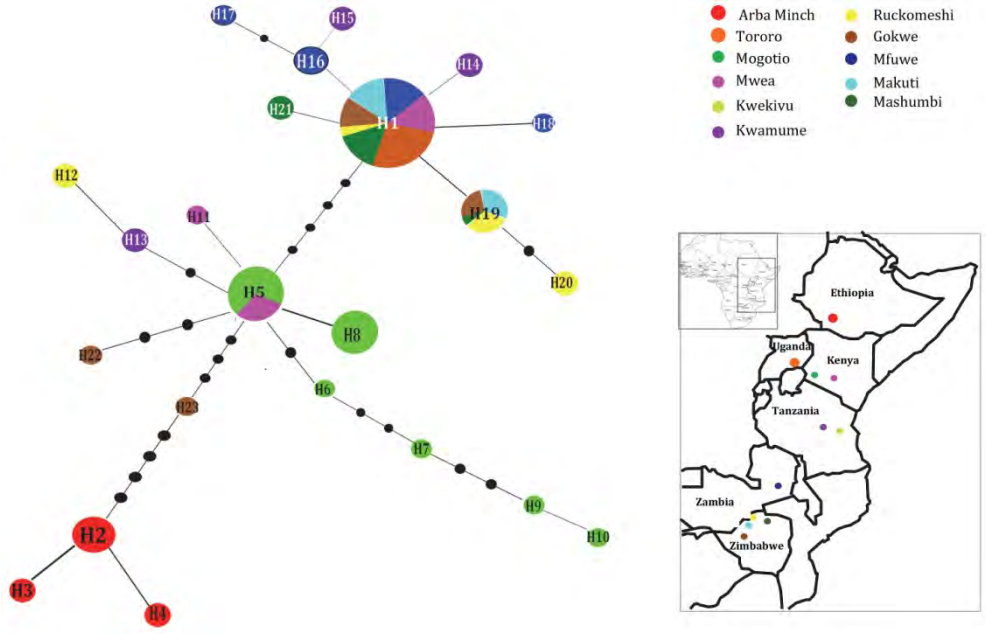


**Figure 3: GpSGHV haplotype network:** The haplotype network generated based on the ML tree for GpSGHV. Each line between the points represents a single mutational step, while haplotypes are represented by a circle whose area is proportional to the number of individuals showing that haplotype. The haplotypes are coloured to match the respective geographical locations where *G. pallidipes* were sampled.

Despite the fact that the GpSGHV haplotypes described in this chapter were not cloned, these haplotypes appear to have very limited genetic heterogeneity, at least as detected in the five PIFs. Such observations have been observed in other dsDNA viruses such as the baculoviruses (See review in Erlandson, 2009 and the references thereof). These observations may not be surprising: the potential for genetic variations in viruses need not necessarily result in high viral diversity because selection factors e.g. virus/host/vector interactions may reduce genetic diversity in populations. Small population diversity and genetic stability may be the rule rather than the exemption. Further studies are required to provide more precise estimates of the GpSGHV diversity. On this note, it is important to stress that the number of the identified virus haplotypes and the frequency of the most prevalent ones, may depend on the size and conserved nature of the analysed genomic target, and on the method of analysis.

### *GpSGHV diversity and potential management strategies*

Symptomatic GpSGHV infections have negative effects on fecundity of laboratory colonies of *G. pallidipes* (Abd-Alla *et al.*, 2007a). There is need to develop viral management that would be applicable not only to *G pallidipes* colonies, but also for colonies of other tsetse species. Potentially, the low GpSGHV diversity presents an opportunity to explore molecular-based viral management strategies that would be applicable in multiple tsetse facilities.

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# Chapter 4

# Proteome and virion components of GpSGHV[3]


## Abstract

In this chapter, the structural components, protein composition and associated aspects of morphogenesis and cytopathology of *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV; family *Hytrosaviridae*) were investigated. Observation of hypertrophied salivary gland cells under the transmission electron microscopy revealed four morphologically distinct viral structures: nucleocapsid, tegument, envelope, and helical surface projections. Nucleocapsids were embedded in virogenic stroma within the nuclei, whereas enveloped virions were restricted to the infected cell's cytoplasm. The cytoplasm of infected cells appeared disassembled, and the plasma membranes disintegrated. Treatment of intact, purified viral particles with 1% Nonidet P-40 efficiently partitioned the virions into envelope and nucleocapsid fractions. The fractions were subsequently separated by 12% SDS-PAGE followed by in-gel trypsin digestion and analysis of the tryptic peptides by liquid chromatography coupled to electrospray and tandem mass spectrometry (LC-MS/MS). Using the MaxQuant program with Andromeda as a database search engine, forty-five viral proteins were identified. Of these, ten and fifteen were associated with the envelope and the nucleocapsid fractions, respectively. Twenty proteins were detected in both fractions, most likely representing tegument proteins. In addition to the virus-encoded proteins, fifty-one host-derived proteins were identified. When subjected to proteinase K protection assay, thirteen of the host-derived proteins were detectable by LC-MS/MS, suggesting that some host-derived proteins may be incorporated into mature GpSGHV particles. These data provide important information about GpSGHV pathobiology, and suggests options for development of future anti-GpSGHV strategies by interfering with virus-host interactions.



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# Introduction

The *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV; family *Hytrosaviridae*) is a rod-shaped, enveloped virus measuring approximately 50 mm in width and 1,000 mm in length (Garcia-Maruniak *et al*., 2009). The virus has a circular dsDNA genome of 190,032 bp that encodes 160 predicted protein-coding open reading frames (ORFs) (Abd-Alla *et al.*, 2010a). To date, salivary gland hypertrophy viruses (SGHVs) have been identified that infect the tsetse fly *G. pallidipes* (GpSGHV) (Jaenson, 1978b), the housefly *Musca domestica* (MdSGHV) (Coler *et al.*, 1993), and probably the narcissus bulb fly *Merodon equestris* Fabricius (MeSGHV) (Amargier *et al.*, 1979). Recently, a virus morphologically similar to the SGHVs was reported in the accessory gland filaments of the braconid wasp *Diachasmimorpha longicuadata* (Luo and Zeng, 2010). GpSGHV and MdSGHV induce similar gross pathologies in infected adult insect hosts, most notably the characteristic salivary gland hypertrophy syndrome (SGH) and significant reduction in reproductive fitness of the adult insects (Abd-Alla *et al*., 2010b; Lietze *et al.*, 2007). Whereas MdSGHV causes only symptomatic infections in the adult house flies (Lietze *et al*., 2011b, 2012), which is characterized by detectable SGH symptoms, tsetse flies infected by GpSGHV exhibit both asymptomatic and symptomatic infection states, with the former being the most widespread in the fly colonies (Abd-Alla *et al*., 2007a).

GpSGHV negatively affects laboratory colonies of *G. pallidipes*, often leading to colony collapse (Abd-Alla *et al.*, 2007a; 2010b). Since the maintenance of healthy, productive tsetse colonies is vital to research and applications of tsetse and trypanosomosis eradication programs, there is need to develop novel strategies to manage GpSGHV infections in tsetse fly mass production facilities. During membrane feeding, one viremic fly deposits up to $10^7$ viral genome copies via salivary secretions in the form of virus particles into a blood meal. The virus particles secreted via saliva have been shown to be infectious *per os* to healthy *G. pallidipes* (Abd-Alla *et al.*, 2010b). Although it is unknown how the virus gets into salivary glands, it is assumed that ingested virions enter via the midgut, transverse the hemolymph-filled hemocoel to reach the glands (Garcia-Maruniak *et al.*, 2009) where they are presumed to reside until transmission to new host. Although it is known that the GpSGHV virion proteome consists of 61 virally encoded proteins (Kariithi *et al.*, 2010), the localization and function of the viral proteins, and their respective contributions to the viral ultrastructure and the infection process are unknown. It is particularly important to determine GpSGHV envelope proteins (likely to be involved in the viral entry into cells), and to know which virion proteins contribute to viral pathobiology as these proteins are possible targets for development of antiviral strategies. In this chapter, the structure of GpSGHV virions is detailed, and a comprehensive repertoire of viral and cellular proteins with their localization within the virion is presented.

# Materials and Methods

## *Electron microscopy of hypertrophied salivary glands*

Hypertrophied salivary glands were freshly dissected from adults of a laboratory colony of *G. pallidipes* flies maintained at the tsetse fly production facility in Seibersdorf, Austria. Freshly dissected glands were immediately fixed (4 h; 4°C) in 2% paraformaldehyde/3% glutaraldehyde in 0.1 M phosphate/citrate (PC) buffer, pH 7.2. The glands were washed, infiltrated with 2.3 M sucrose in PC buffer (16 h; 4°C), and cryo-fixed by plunging into liquid ethane at -160°C using a Reichert KF80 plunger. Cryo-sections (80 nm thick) were cut at -110°C with a Leica Ultracut S microtome equipped with FCS cryo-system, mounted on formvar-coated copper grids (100 mesh), and negatively stained with 3% ammonium molybdate, pH 6.5 and air-dried. Images were recorded with a Gatan 4K CCD camera on a JEOL 2100 transmission electron microscope (TEM) equipped with a LaB6 filament operating at 200 kV.

## *Electron microscopy of GpSGHV particles*

Salivary glands were gently squashed in 1:1 diluted PC buffer, and extracts incubated on formvar- and carbon-coated copper grids (100 mesh) and stained with 1% uranyl acetate, pH 3.7. Similar specimens were prepared from purified virus suspensions (see next section). For electron tomography, fiducial gold markers (10 nm) were included in the viral extract and series of 2 x-binned images recorded with SerialEM (Mastronarde, 2005) at tilt angles from -65 to +65 degrees with increments of one degree. The series of tilted projection images were converted into 3D tomograms using the IMOD program (Kremer *et al.*, 1996).

## *Virus purification*

Three replicates of viral extractions were conducted on twenty-five pairs each of hypertrophied salivary glands freshly dissected from 10-day old adult *G. pallidipes* flies. The glands were immediately disrupted by two strokes of a glass/Teflon homogenizer (on ice) in 1 ml of homogenization buffer (50 mM HEPES pH 8.0, 10 mM Ficoll PM400 [GE Healthcare], 2 mM EDTA, protease inhibitors [ROCHE]). The volumes were brought to 2 ml and clarified by three times centrifugation (7,500 × g; 10 min; 4°C). The supernatants were pooled and layered onto 5 ml of a 10-40 % (w/v) Ficoll PM400 discontinuous density gradient and ultra-centrifuged (25,000 × g; 1h; 4°C). The virus band was collected, re-suspended in 50 mM HEPES buffer, pH 8.0 and ultra-centrifuged (60,000 × g; 60 min; 4°C). The resultant pellet was allowed to dissociate into 1 ml of 50 mM HEPES buffer, pH 8.0 (o/n; 4°C). Integrity of purified virions was checked by negative staining using a JEOL 2100 TEM (Kariithi *et al.*, 2010).

## *Fractionation of GpSGHV virions into envelope and nucleocapsids*

Purified viral particles were incubated (30 min; RT) in 250-µl reaction volumes with Nonidet P-40 lysis buffer (1 % NP-40, 50 mM Tris, pH 8.0, 137 mM NaCl, 10 % glycerol and 2 mM EDTA). The NP-40-treated virions were layered onto a 5 ml 10-60% (v/v) glycerol gradient and ultra-centrifuged (110,000 × g; 1 h; 4°C). The viral envelope fraction was collected from the top 2 ml of the gradient. The pellet was subjected to a second round of 1% NP-40 extraction and glycerol ultracentrifugation to ensure complete removal of virion envelopes. Purity of nucleocapsids was checked by negative-staining TEM as described above. The envelope fraction was precipitated with trichloro-acetic acid (TCA) (overnight; 4°C). The TCA-precipitated proteins were recovered by centrifugation (20,000 × g; 15 min) and the TCA was neutralized by three washes with ice-cold acetone. The pellets were dried and re-suspended in 10 mM Tris, pH 8.0.

## *Identification of GpSGHV structural proteins by LC-MS/MS*

Portions of the envelope and nucleocapsid fractions were treated with lysis buffer (8 M urea, 4 mM $CaCl_2$, 0.2 M Tris-HCl, pH 8.0) and separated by 12% SDS-PAGE. The gel was stained with the Colloidal Staining Kit (Invitrogen). The middle sections of entire gel lanes were excised and the gel sections cut into small pieces (~ 1 $mm^3$). In-gel trypsin digestions were performed and the resultant peptides were analysed LC-MS/MS (Kariithi *et al.*, 2010). The LC-MS/MS data were analysed by the MaxQuant software package (version 1.2.2.5) (Cox *et al.*, 2011; Cox and Mann, 2008) with the following constructed databases: a GpSGHV ORF database (http://www.uniprot.org/), a contaminant database containing sequences of common contaminants (BSA [P02769, bovine serum albumin precursor], Trypsin [P00760, bovine], Trypsin [P00761, porcine], Keratin K22E [P35908, human], Keratin K1C9 [P35527, human], Keratin K2C1 [P04264, human] and Keratin K1CI [P35527, human]), and a *G. m. morsitans* database (http://www.sanger.ac.uk/). Proteins were identified with the MaxQuant software using default settings for the Andromeda search engine (Cox *et al.*, 2011) except that extra variable modifications were set for de-amidation of N and Q. Peptides and proteins with a false discovery rate (FDR) of less than 1%, and proteins with at least two peptides of which at least one was unique were accepted for further analyses. The peptide peak intensities (abundances) from the MS/MS runs were normalized to the total peak intensity based on all identified peptides in the analysis. The normalized peptide abundances (from the three biological sample replicates) were used to perform a Student's *t*-test statistical analysis by Perseus module (version 1.2.0.17), which is available with at the MaxQuant platform. The normalized peptide abundances were plotted in MA (minus versus average) plot, showing the average log abundance on the *x*-axis and the $\log_{10}$ fold change on the *y*-axis.

## *Localization of GpSGHV envelope and nucleocapsid proteins*

Another portion of the envelope and nucleocapsid fractions of the purified viral particles were separated by SDS-PAGE (12%) and transferred onto Immobilon-P (Millipore) membranes by electrophoresis according to standard protocols. Membranes were blocked by overnight incubation with 5% non-fat milk powder and 0.05% Tween-20 in TBS buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl) at 4°C. The membranes were then incubated (1 h; RT) with rabbit polyclonal antibodies against proteins encoded by GpSGHV ORF1 (P74) or ORF10 as the primary antibodies (diluted 1:1000). Membrane were then washed three times with TBS-T buffer, and further incubated (1 h; RT) with alkaline phosphatase-conjugated polyclonal goat anti-rabbit IgG antibody (Sigma; diluted 1: 2000) as the secondary antibody. Blots were developed with NBT/BCIP (Sigma).

## *Verification of incorporation of cellular proteins into GpSGHV particles*

Purified GpSGHV particles were incubated for 30 min at 37°C with 0.08 µg protease K (protK) (Invitrogen) per microgram of total protein (Moerdyk-Schauwecker *et al.*, 2009). ProtK activity was stopped by addition of PMSF to final concentration of 5 mM followed by incubation on ice for 15 min. Contaminating vesicles were removed by passing a portion of the protK-treated material through a 5 ml 20% Ficoll cushion by ultra-centrifugation (60,000 × g; 1 h; 4°C). The non-treated and protK-treated samples were separated by 12% SDS-PAGE gel, followed by in-gel trypsin digestion and LC-MS/MS analyses as described above. A portion of the protK-treated sample was subjected to Western blotting using mouse anti-tubulin-α (Sigma; clone DM1A, T6199), mouse monoclonal cytoplasmic actin (Sigma; clone 10-b3, A0480), mouse monoclonal IgG anti-ubiquitin (Ub-P4DI, cs-8017; Santa Cruz Biotechnology, Inc.), or rabbit polyclonal anti-myosin (MYH; H-300, sc-20641, Santa Cruz Biotechnology, Inc.) as the primary antibodies (diluted 1:3000). Alkaline phosphatase-conjugated anti-mouse IgG (Sigma; diluted 1:2000) was used as secondary antibody using the above-mentioned conditions.

## *Phosphorylation and glycosylation analyses of GpSGHV proteins*

To investigate the phosphorylation status of the GpSGHV proteins, proteins prepared from the virus fractions described above were electrophoresed on 12% SDS-PAGE gels and transferred Immobilon-P membranes for Western blotting according to standard protocols. The membranes were blocked with 1% skim milk/0.05% Tween-20 in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, and 1.76 mM $KH_2PO_4$, pH 7.4) (overnight; room temperature). For the Western blotting mouse monoclonal anti-phospho-serine/threonine/tyrosine (Thermo Scientific; diluted 1:150) and anti-mouse IgG alkaline phosphatase (Sigma; diluted 3000) as the primary and secondary

antibodies, respectively following the supplier's instructions.

Glycosylation potential of GpSGHV proteins was performed using the Pro-Q Emerald 300 Glycosylation Gel and Blot Stain Kit (Molecular Probes, Invitrogen) according to manufacturer's protocol, with slight modifications. Briefly, GpSGHV fraction described above electrophoresed on a 12% SDS-PAGE gel followed by oxidization with periodic acid for 30 min. After washing with 3% glacial acetic acid to remove residue periodate, the gels were incubated in Pro-Q Emerald 300 stain solution (diluted 25-fold into staining buffer) for 90 min and subsequently washed. Stained gels were stained with SYPRO Ruby protein gel stain to detect the glycoproteins by visualization using 300 mm UV illumination on glycoproteins.

### *Functional and structural characterization of GpSGHV proteins*

Identified viral proteins were annotated using Blast2GO software version 2.5.0 (Conesa *et al.*, 2005). Protein motifs were analysed using ExPASy PROSITE database (http://www.expasy.org). The numbers of trans-membrane (TM) helices were predicted by TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), and signal peptide sequences were predicted using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0/). As complementary to analyses of phosphorylation and glycosylation potential of the identified proteins, predictions were also derived by computational analysis using NetPhos 2.0 CBS Prediction server (http://www.cbs.dtu.dk/services/NetPhos/) with a threshold value set at 0.7, and NetGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/), respectively.

## Results and Discussion

### *Signature features of GpSGHV virions in infected host cells.*

Electron microscopy of cryo-sections of *G. pallidipes* hypertrophied salivary glands showed nucleocapsids embedded in virogenic stroma within the infected cells' nuclei (**Fig. 1 a, panel i**), presumably induced by GpSGHV infection. The induction of the formation of the virogenic stroma has been reported in other nuclear-replicating dsDNA viruses. For instance, in baculoviruses, the packaging of virus particles has been demonstrated to occur in the virogenic stroma, where empty capsids assemble in the pockets between chromatin-like filaments, and then the capsids filled with DNA acquired from the virogenic stroma (Fraser, 1986; Young *et al.*, 1993).

A key question is how the virus acquires its envelope. Enveloped viruses are known to acquire their envelopes through various mechanisms. For instance, white spot syndrome virus (WSSV, *Nimaviridae*) acquires the envelope within the nucleus (Xie *et al.*, 2006), while herpes viruses are enveloped by budding either through the nuclear

membrane or through trans-Golgi membranes (Johnson and Baines, 2011). Other herpes viruses are enveloped entirely in the cytoplasm (Tandon and Mocarski, 2011).

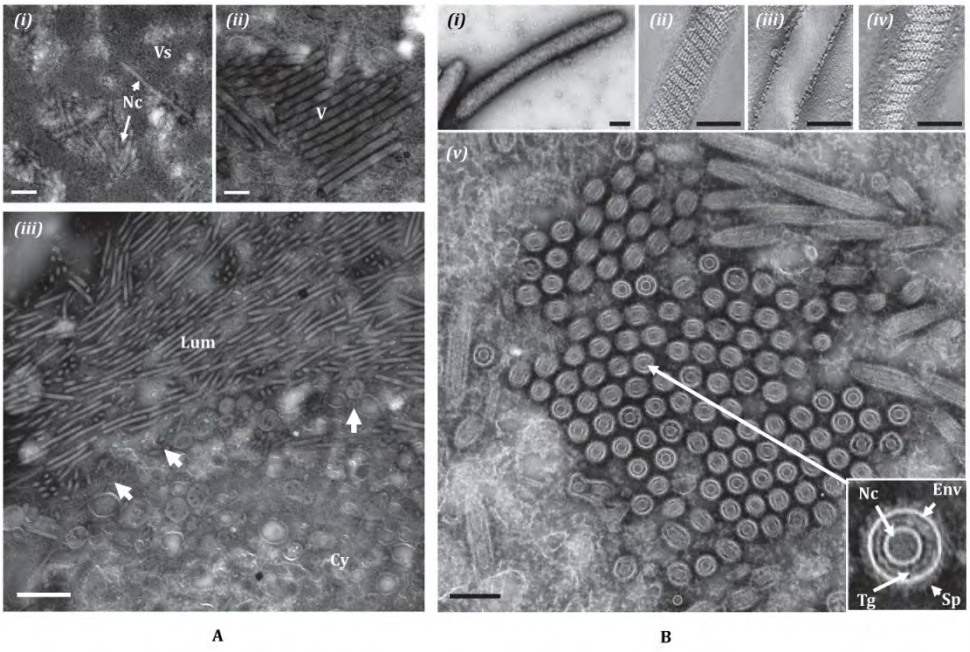


**Figure 1: Ultrastructural analysis of infected salivary glands and GpSGHV virions by TEM:** (A) Cryo-sections through hypertrophied salivary gland cells. Nucleocapsids (Nc) are embedded in virogenic stroma (Vs) present in nucleus (i), while enveloped virions (V) are observed in cytoplasm (Cy) (ii) and in the glands' lumen (Lum) (iii). Notice that the infected cytoplasm appears jumbled, plasma membrane appears disintegrated (indicated with arrowheads) and the lumen full of enveloped virions (iii). Bars represent 200 nm (i, ii), and one µm (iii). (B) Electron micrographs of negatively stained GpSGHV enveloped virions. The surface of a mature virion consists of regular helically arranged surface projections (i). The top, middle and bottom views of the virion particle shown in (i) are also shown (ii to iv respectively). (v) Shows a cryo-section through a group of virions in an infected cell cytoplasm. The inset show high magnification image of the cross section through a virion particle, revealing the nucleocapsid (Nc) tegument (Tg), envelope (Env) and surface projections (Sp). Bars represent 100 nm (i to iv) and 200 nm (v).

Many enveloped vertebrate viruses (e.g. ortho- and paramyxoviruses, retroviruses) and invertebrate viruses (baculovirus budded viruses) acquire their envelopes by budding through the plasma or nuclear membranes. The data presented in this chapter indicated that naked GpSGHV nucleocapsids were abundant in the nucleus, while enveloped virions were restricted to the cytoplasm of the infected cells (**Fig. 1 a, panel ii**). Occasionally, some naked nucleocapsids were also observed in the cytoplasm. Further, in all the examined sections, there was no evidence of virus budding through the plasma membrane into the salivary gland lumen. These

observations appear to suggest that the GpSGHV virions might be released intra-cellular into cell compartments and subsequently acquire the envelope in the cytoplasm. This is in agreement with recent evidence that the nucleocapsids of MdSGHV egress from the nucleus via nucleopore complexes for cytoplasmic envelopment (Boucias *et al.*, 2013a). However, a remarkable difference is that unlike GpSGHV, the MdSGHV particles migrate to and bud out of the plasma membrane bordering the salivary gland lumen (Boucias *et al.*, 2013a; Lietze *et al.*, 2011a). The lumen of GpSGHV infected salivary glands was filled with closely packed arrays of rod-shaped, enveloped virions (**Fig. 1 a, panel iii**). It appears that these virions are continuously shed during membrane feeding in tsetse colonies, and are infectious *per os* to healthy flies (**Chapter 2**). Further, infected salivary gland cells appeared disarrayed and extended into adjoining lumen with the plasma membranes disintegrated (**Fig. 1 a, panel iii**), which may imply that GpSGHV virions egress from the infected cell via disintegration (or perhaps rupture) of the plasma membranes.

The GpSGHV nucleocapsid core consists of a thin dense layer surrounding a central, higher density area, suggesting that the core is not hollow. Assuming an equal distribution of the super-helical DNA in the nucleocapsid, the super-helicity of GpSGHV DNA (190 kbp; 900 nm-long nucleocapsid) is approximately half that of the average baculovirus (130 kbp; 300 nm-long nucleocapsid) (Jehle *et al.*, 2006). Negative staining TEM and electron tomography of enveloped virions extracted from freshly excised glands showed a helical arrangement of elongated surface projections. These surface projections measured ~ 13 nm in length and had a periodicity of approximately 15 nm (**Fig. 1 b, panels' i-iv)**. Surface projections have been reported in other viruses such as the vesicular stomatitis virus (VSV) (Cartwright *et al.*, 1969) and several poxviruses (Hiramatsu *et al.*, 1999b). In VSV, the surface projections are composed of cellular-derived proteins or macromolecules and virus-specific antigens; enzymatic removal of these substructures prevented attachment of VSV to susceptible cells (Cartwright *et al.*, 1969). In another study, De Giuli *et al.,* (1975) suggested that the surface projections in some strains of Rous sarcoma virus (RSV) are essential for the interaction with cellular receptors to permit initiation of the virus infection process.

The data presented in this chapter did not establish the identities of the composition of the GpSGHV surface projections. However, it is tempting to speculate that the surface projections observed in GpSGHV particles are made up of polymeric structures of viral proteins. Some of the host-derived proteins (see below) may be present in the GpSGHV surface projections. GpSGHV virion contains an internal core with an average diameter of 40 nm; between envelope and nucleocapsid lies an amorphous, electron-dense proteinaceous matrix of approximately 10 nm thick (**Fig. 1 b, panel v**). This structure is likely to constitute the GpSGHV tegument, which may form a structural link between the nucleocapsid and the envelope.

## *Purification and fractionation of GpSGHV particles*

For a comprehensive analysis of the full repertoire of viral proteins, purity and integrity of viral preparations are critical. Initial purification of GpSGHV particles using sucrose gradients resulted in total loss of the virus envelope, leading to the erroneous conclusion that GpSGHV is a non-enveloped DNA virus containing only twelve polypeptides (Odindo *et al.*, 1986). Although the purification of the virus was later improved by use of nycodenz gradient centrifugation (Abd-Alla *et al.*, 2007a; Kariithi *et al.*, 2010), the integrity of the viral particles was still compromised. In the current study, an improved GpSGHV purification protocol was developed using the high molecular sugar Ficoll, prepared in an organic buffer (HEPES) at high pH (pH 8.0), and supplemented with a cocktail of protease inhibitors. The improved GpSGHV purification protocol resulted in preservation of the rod-shape GpSGHV virions with an intact envelope surrounding the viral particles (**Fig. 2a**).

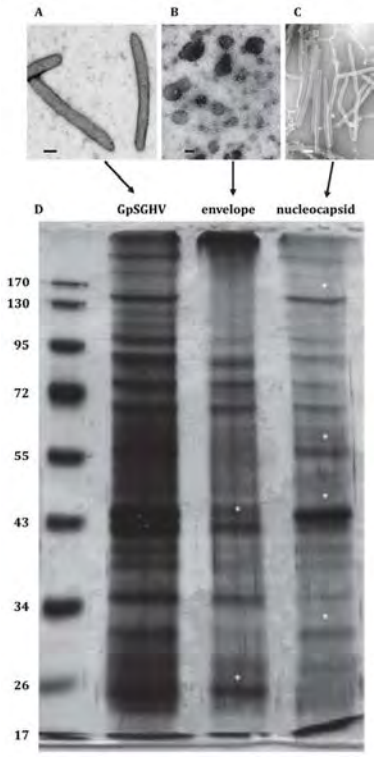


**Figure 2: Purification and fractionation of GpSGHV particles:** TEM micrographs of high-quality GpSGHV virions after purification using 10-40% Ficoll gradient centrifugation (A). Treatment of the purified virions with 1% NP-40/137 mM NaCl buffer resulted to efficient removal of envelope (B) from the nucleocapsids (C). Protein profiles of intact virions, envelope and nucleocapsid components of purified virions (D). Bars represent 100 nm (A - C).

Treatment of these particles with 1% NP-40/137 mM NaCl buffer resulted in efficient separation of the envelope and the nucleocapsid components (**Fig. 2b and 2c**). Silver staining of SDS-PAGE gels of NP-40-treated virions showed several dominant bands in

the nucleocapsid fraction and in the envelope fraction of approximately 40 and 26 kDa (**Fig. 2d**). Several other proteins observed in the intact virions were associated with either the nucleocapsid or the envelope fractions with varying intensities. A high molecular weight smear was observed close to the top of the resolving gel in the envelope fraction (**Fig. 2d**) and may be an indication for covalently modified glycoproteins.

## *Phosphorylation and glycosylation analyses of GpSGHV proteins*

Phosphorylation is one of the common posttranslational modifications of proteins. Phosphoproteins have been reported in the structural proteins of the members of several viruses; these phosphoproteins could be either structural or non-structural. For instance, the HSV-1's *tras*-acting proteins IE1/α0, IE2/α27, IE3/α4, IE4/α22 (Ackermann *et al.*, 1984), DNase (Banks *et al.*, 1985), the large subunit of ribonucleotidase reductase (Preston *et al.*, 1984), and a structural component of the tegument (Campbell *et al.*, 1984). Phosphorylations may be necessary for the interaction of viral nucleic acids, subsequent assembly (Sen *et al.*, 1977; Sen and Todaro, 1977), uncoating or disassembly (Lackmann *et al.*, 1987; Witt *et al.*, 1981) of the virions, interaction between viral proteins and host DNA (Scheidtmann *et al.*, 1984), as well as regulation of transcriptional activity (Hsu *et al.*, 1982; Hsu and Kingsbury, 1985; Kingsford and Emerson, 1980).

In the current study, Western blots using polyclonal antibody directed against the product of GpSGHV ORF 10 showed multiple bands both in the nucleocapsid and envelope fractions, the most prominent of which were present in the nucleocapsid fraction (**Fig. 3A**). As can be observed in the multiple bands in figure 3A, the antibody against ORF10 appears to be either non-specific. Alternatively, this protein may have modified forms *in vivo* such as acetylation, methylation, myristoylation etc. Further, the multiple bands could be a reflection of the disadvantages of Western blotting, whereby incidental or oxidation of proteins may result in multiple bands appearing during sample preparation.

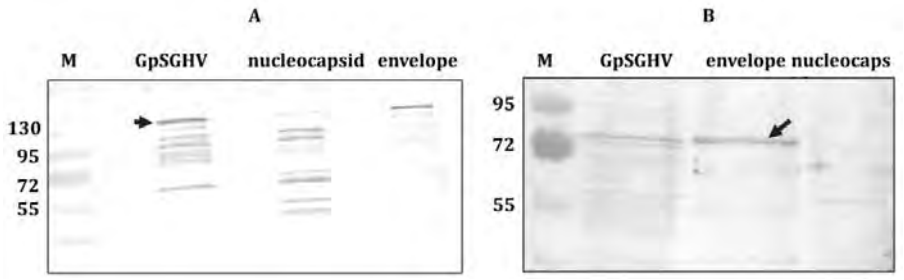


**Figure 3: Western blot analysis of the GpSGHV virion proteins:** The western blot was done using anti-rabbit serum against proteins encoded by GpSGHV ORF10 (A) and ORF1 (P74) (B).

Western blot analysis confirmed the presence of the marker for the GpSGHV envelope (the P74 protein) in the envelope fraction (**Fig. 3B**) and its absence in the nucleocapsid fraction. Further studies are needed to determine GpSGHV phosphoproteins, what roles are played by these proteins in the virus morphogenesis, and kinases responsible for their phosphorylation.

Intact GpSGHV virions, the envelop and nucleocapsid fraction proteins were resolved by SDS-PAGE. The presence of proteins modified by glycosylation was tested using a Pro-Q Emerald fluorescent carbohydrate-specific staining. Several protein bands were detected as carbohydrate-positive (**Fig. 4**).

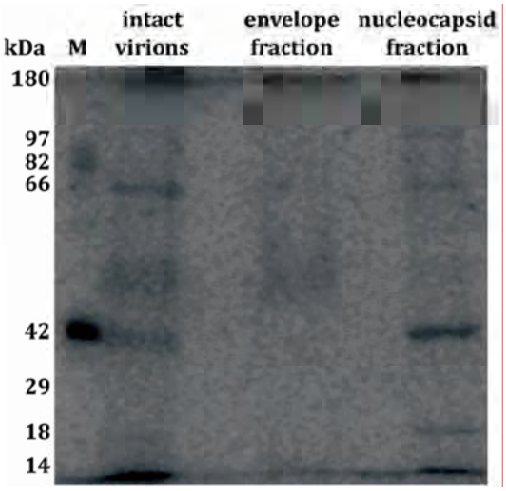


**Figure 4**: **Analysis of the glycosylation status of purified GpSGHV virion proteins:** Purified intact virus virions and the envelop and nucleocapsid proteins were separated on a 12% SDS-PAGE gels, followed by staining with the Pro Q Emerald 300 Glycoprotein Gel and Blot Stain Kit according to manufacturer's instructions. M denotes the SYPRO Ruby protein gel stain. The glycoproteins were visualized by illumination with 300 mm UV light.

Although this study did not verify the identity of the glycosylated GpSGHV proteins, the detection of glycosylation signals indicate that the virus has several glycoproteins. To complement this limitation, glycosylation of the GpSGHV proteins were predicted *in silico*, using the strict consensus amino acid sequon (Asn-X-Ser/Thr-Y (for *N*-linked glycosylation) and Ser or Thr for *O*-linked glycosylation (Shakin-Eshleman *et al.*, 1996). The predicted GpSGHV glycoproteins are summarized in **Table 1**. The 'X' in the sequon Asn-X-Ser/Thr could be any amino acids except Pro. It should be noted that although the occurrence of the sequon does not always guarantee glycosylation, the amino acid following the sequon (position 'Y') determines the efficiency of glycosylation. In general, positively-charged amino acids (Lys, Arg and His) or small amino acids (Gly, Asp, Ala and Asn) at position X favour glycosylation; Arg, Thr, Ser and Cys are the most favourable at either positions X or Y (Mellquist *et al.*, 1998). Based on the amino acids present in the Asn-X-Ser/Thr-Y sequon of GpSGHV proteins (marked with '§' and '¥'in **Table 1**), it is highly likely that the virus has many glycosylated proteins.

**Table 1: Summary of potentially glycosylated GpSGHV proteins predicted by the NetGly 1.0 server:** The *N*-linked glycosylation (the most common way for glycosylation of viral proteins) has the strict consensus amino acid sequon Asn-*X*-Ser/Thr (see Shakin-Eshleman *et al.*, 1996), where *X* is any amino acid except Pro. The *O*-linked glycosylation is on Ser or Thr, and does not require a consensus sequence. Note that only proteins identified by LC-MS/MS in this study (shown in **Table 2**) are shown in the table.

| ORF No. | UniProt IDs | Mol. mass [kDa] | Position of predicted *N*-glycosylation predicted site(s) § | Position of predicted *O*-glycosylation site(s) |
|---|---|---|---|---|
| 1 (P74) | B0YLF6 | 81.4 | 156 NR§TD | T 163 |
| 10 | B0YLG4 | 127.0 | 157 NISL | T 296 |
| 36 | B0YLJ0 | 13.8 | 16 NVSD | - |
| 38 | B0YLJ2 | 136.7 | 25 NT§TN; 262 NETT§; 379 NVSS§; 586 NG§TL | T 777, T 936, T 936, T 1110-1129 |
| 39 | B0YLJ3 | 37.7 | 46 NVSS§ | S/T 121-197 |
| 44 | B0YLJ8 | 42.8 | 167 NG¥TK§ | - |
| 45 | B0YLJ9 | 201.1 | 277 NYTD; 337 NS§SS§; 1455 NR§TS§; 1522 NT§TL; 1697 NITN | T 843, T 955 |
| 46 | B0YLK0 | 61.5 | 15 NC§SE; 180 NS§TK§; 252 NS§TV | - |
| 47 | B0YLK1 | 47.2 | 175 NITN | - |
| 50 | B0YLK4 | 32.7 | | T 23 T27; T 254; T285-287 |
| 53 (PIF-2) | B0YLK7 | 40.2 | 6 NFTL; 17 NAST§ | - |
| 61 | B0YLL5 | 57.4 | 276 NA¥TI; 294 NVTD | - |
| 62 | B0YLL6 | 512.1 | 1841 NR§TS§; | T 306 -309 |
| 69 | B0YLM3 | 30.9 | 202 NITR§; 212 NSSI; 247 NMSD; | - |
| 71 | B0YLM5 | 72.0 | 83 NVSL | - |
| 72 | B0YLM6 | 31.8 | 116 NITN | - |
| 83 | B0YLN7 | 81.6 | 20 NITY; 584 NITT§ | - |
| 85 | B0YLN9 | 30.1 | 67 NVTK | - |
| 86 | B0YLP0 | 70.2 | 57 NR§SL; 370 NISK§ | - |
| 88 | B0YLP2 | 77.8 | 368 NK§TI; 584 NMSL | T 212 |
| 94 | B0YLP8 | 32.7 | 61 NISN; 69 NVSR§ | - |
| 96 | B0YLQ1 | 44.4 | 151 NETS§ | - |
| 97 | B0YLQ0 | 43.5 | 142 NK§TL; 176 NMTS§ | T 379; T 385; S 388; T 393 |
| 102 (PIF-1) | B0YLQ6 | 76.1 | 194 NC§SR§; 464 NG§TI | T 433 |
| 104 | B0YLQ8 | 77.9 | 371 NT§TR§; 450 NVTT§ | - |
| 106 | B0YLR0 | 55.1 | 375 NITK§ | - |
| 108 | B0YLR2 | 63.9 | 501 NK§TM; 510 NESL | - |
| 112 | B0YLR6 | 19.1 | - | T 27; T 28; S 34; T 35 |
| 154 | B0YLV8 | 40.1 | 16 NIST§; 155 NETK§; 279 NVSN | - |

§ The positively charged amino acids that at position X and/or Y in the sequon Asn-X-Ser/Thr-Y that are known to favour (efficiency of) glycosaylation; ¥ Small amino acid residues at position X that may also favour glycosylation.

Assuming that GpSGHV has many glycoproteins as argued above, then these glycoproteins may have important roles in the virus pathobiology. The role of glycosylation in viral proteins is well documented. Glycosylation of viral proteins may impart survival and virulence advantages to viruses (Vigerust and Shepherd, 2007). For instance, in influenza virus for instance, glycosylation modulates entry into host cells (Klenk *et al.*, 2002) and tissue tropism (Deshpande *et al.*, 1987; Zambon, 1999), while in HIV-1, glycosylation plays crucial roles in evasion of host immune system (Sagar *et al.*, 2006). Glycosylation of the premembrane (prM) and the envelope (E) proteins of WNV plays has roles in viral replication and (Hanna *et al.*, 2005; Shirato *et al.*, 2004), while the envelope glycoproteins E1 and E2 of HCV play essential functions

in intracellular transport (Goffard *et al.*, 2005; Goffard and Dubuisson, 2003). In new and emerging viruses e.g. Ebola, Hendra and SARS-CoV, *N*-linked glycosylation has roles in proteolytic protein processing (see reviews in Bossart *et al.*, 2005; Lin *et al.*, 2003; Oostra *et al.*, 2006). Further investigations are required to verify which of the GpSGHV proteins are indeed phosphorylated, and what roles these glycoproteins potentially play during the viral infection process.

## *Prediction of the distribution of identified proteins on GpSGHV particle*

Based on FDR cut-off of < 0.1, forty-five virion proteins were identified by LC-MS/MS analysis of the envelope and nucleocapsid fractions (see **Table 2**). Of the forty-five GpSGHV proteins, ten were found only in the envelope fraction, fifteen proteins were found only in the nucleocapsid fraction and twenty proteins were measurably present in both the envelope and the nucleocapsid fractions. A distribution of the identified GpSGHV and cellular proteins is shown in **Figure 5**.

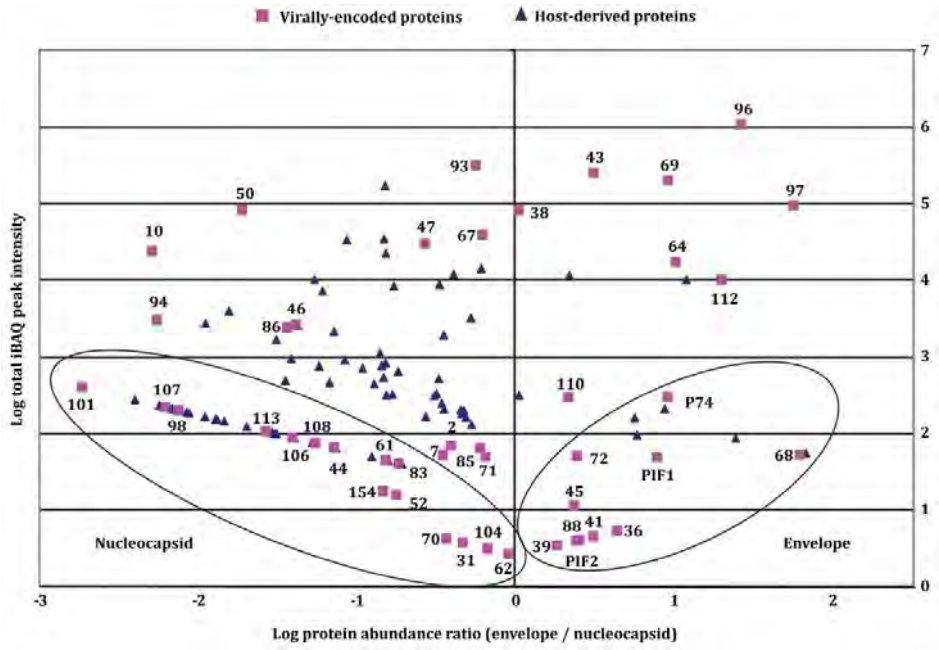


**Figure 5: Abundance distribution of GpSGHV-encoded proteins (squares) and virion-associated cellular proteins (triangles) identified by LC-MS/MS**: Large circles enclose the proteins that were measured only in either the envelope or the nucleocapsid fractions, while the rest were detectable in both fractions. The numbers correspond to the GpSGHV ORFs. iBAQ denotes intensity-based absolute quantification. Refer to **Table S1** (Available JGV Online: Kariithi *et al.*, 2013b) for identities of the cellular proteins detected in the envelope and nucleocapsid fractions.

Proteins detected only in the nucleocapsid and envelop fractions are circled. It should be noted that the proteins shown in Table 2 and Figure 5 are only those that were detectable in all the three biological replicates used in this study; for construction the protein distribution map, the proteins were filtered with high stringency i.e. at least 2 unique peptides per protein.

One of the questions to be addressed in this study was how the identified GpSGHV proteins are distributed in the structural components of the viral particle, i.e. the nucleocapsid, the (presumed) tegument and the envelope. It should be noted that the current study only predicts potential localization of the identified proteins on the virus particle. More evidence than presented in this chapter is needed to make solid conclusions. One of the ways to do this is for instance, to use NP-40 fractionation in combination with various concentrations of NaCl to separate intact GpSGHV virions into distinct fractions such that each fraction contains the envelope proteins only, envelope and tegument proteins, tegument and nucleocapsid proteins, or nucleocapsids proteins only (Tsai *et al*., 2006). Following such a fractionation protocol, the data obtained from subsequent gradient SDS-PAGE protein profiles, LC-MS/MS, immunogold electron microscopy, and Western blotting, distinct proteins can be localized precisely in each of the fractions. These arguments notwithstanding, it is still possible to predict potential localization of the identified proteins on the GpSGHV particle based on the data presented in this chapter. First of all, the integrity of fractionation of GpSGHV virions was confirmed by TEM such that purified virions had intact envelops, the double NP-40 treated nucleocapsids were free of viral envelops and the envelope fraction was free of nucleocapsids. This means that proteins in the nucleocapsid and envelop fractions that were identified by LC-MS/MS are highly likely to be bonafide components of the respective viral components (See **Fig. 5**). Secondly, evidence obtained from studies in in other viral systems such as in the case of herpes viruses have demonstrated that inner tegument proteins interact closely with the nucleocapsid while the outer tegument proteins interact with the cytoplasmic tails of viral envelope proteins during the cytoplasmic envelopment process of the virus (Guo *et al*., 2010). Based on the protocol used to fractionate GpSGHV virions in this study, the proteins detected in both the envelope and nucleocapsid fractions may be considered tegument proteins with close association with the respective fractions in which they were detected. The tegument proteins may be thought to link the inner nucleocapsid to the envelop viral structural components. On this note, of the twenty proteins that were found to be associated with both the nucleocapsid fractions, five (encoded by ORFs 50, 10, 94, 46 and 86) were found to be much more abundant in the nucleocapsid fraction than in the envelope fraction (**Fig. 5**). Similarly, five other proteins (encoded by ORFs 96, 97, 69, 64 and 112) were much more abundant in the envelope than in the nucleocapsid fraction. Further, previous immunogold labelling of GpSGHV virions with antibodies against the C-terminal fragment of GpSGHV ORF10

gave indications that ORF10 product may be a major component of the GpSGHV virions (Kariithi *et al*., 2010; **Fig. 6**), which in this case appears to be closely associated with the viral nucleocapsid as can be inferred from **Fig. 5**. It should be noted that in figure 6, the GpSGHV virions were purified by nycodenz, which led to depletion of the viral envelope. Attempts on immunogold labelling on the viral preparations using antibodies against ORF96 were unsuccessful.

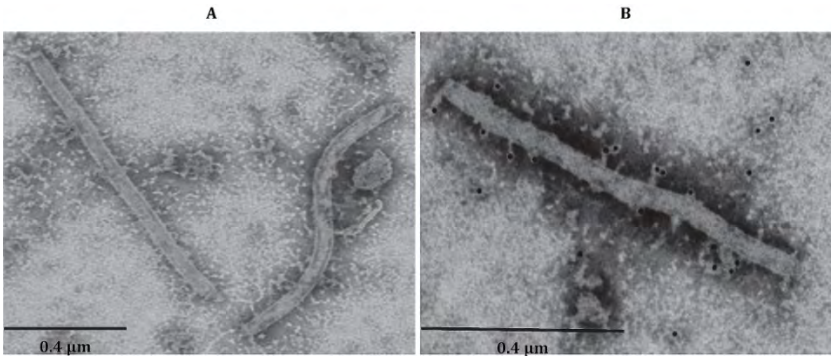


**Figure 6: TEM immunogold labelling of nycodenz-purified GpSGHV particles:** (A) GpSGHV virions labelled with pre-immune serum and (B) rabbit antiserum against C-terminal fragment of GpSGHV ORF10 (Figure adapted from Kariithi *et al*., 2010)

Based on the above-mentioned observations, almost 50% of the identified GpSGHV structural proteins could be predicted to be constituents of the GpSGHV tegument (**Table 2**). This is similar to the situation in human cytomegalovirus (HCMV), in which 50% of the viral proteins are localized in the viral tegument (Varnum *et al.*, 2004). Noteworthy, three of the GpSGHV envelope proteins are homologs of the baculovirus occlusion-derived virus (ODV) envelope proteins P74, PIF-1 and PIF-2, which in baculoviruses are essential during the primary infection in midgut epithelium cells of the host (Slack and Arif, 2007). Potentially, the GpSGHV PIF homologues have similar *per os* infection roles in tsetse midguts. After ingestion, SGHVs find their way to the salivary glands, thereby causing distinct hypertrophy of the gland tissues (Garcia-Maruniak *et al.*, 2009). It is not known how these viruses induce hyperplasia of the infected glands. However, it is noteworthy that the GpSGHV PIF-1 sequence contains an EGF-like domain, SP, TMs, and multiple tyrosine kinase phosphorylation sites (**Table 2**). Proteins containing EGF-like domains are implicated in initiating a tyrosine kinase-mediated signalling cascade that culminates in recruitment of the evolutionally conserved MAPK pathway and results in growth/differentiation signals (Alroy and Yarden, 1997). Interestingly, it has been demonstrated that the fowl poxvirus ORF FPV211 product, which also contains these structural features contributes to the hyperplasia of FPV-infected tissues (Afonso *et al.*, 2000). Whether PIF-1 plays a role in tsetse salivary glands hyperplasia remains to be investigated.

**Table 2: GpSGHV virion proteins:** Forty-five GpSGHV proteins identified by LC-MS/MS. The proteins listed in this table were only those that were detectable in all the three biological replicates and were confirmed by at least two unique peptides per protein (see materials and method). Note that the potentially phosphorylated proteins (marked with asterisks were performed *in silico* at the NetPhos 2.0 CBS Prediction server).

| Predicted Classification¥ | ORF No. | UniProt IDs | Mol. mass [kDa] | Coverage [%] | Peptides identified | Homology to cellular proteins and/or proteins in other viruses | Signature domain(s)/Motifs (amino acid regions harbouring the motifs/domains) | Functional annotation (References) |
|---|---|---|---|---|---|---|---|---|
| Envelope components | 1* | B0YLF6 | 81.4 | 11.8 | 7 | PIF-0 (P74), *S. pectinicornis* NPV | SP; isoleucine-rich activation motif (648-686) | Oral infection (Peng *et al.*, 2010); Viral replication (Chazal and Gerlier, 2003); Tissue-specific gene expression (Attardi and Tjian, 1993; Kuzio *et al.*, 1989) |
| | 88 | B0YLP2 | 77.8 | 8.7 | 6 | | | |
| | 102* | B0YLQ6 | 76.1 | 15.5 | 8 | PIF-1, *Neodiprion abietis* NPV | TM, SP; Epidermal growth factor-like domain; multiple tyrosine kinase phosphorylation sites (248-254, 273-280, 418-489 and 593-599), | Oral infection (Peng *et al.*, 2010); Viral replication (Chazal and Gerlier, 2003); MAPK pathway/growth or differentiation signalling (Afonso *et al.*, 2000; Alroy and Yarden, 1997) |
| | 41 | B0YLJ5 | 48.8 | 13.6 | 5 | ORF MSV214, *M. sanguinipes* EPV | SP, SCG gene family protein | |
| | 53* | B0YLK7 | 40.2 | 14.2 | 4 | PIF-2, *G. bimaculatus* nudivirus | SP | Oral infection (Peng *et al.*, 2010); Viral replication (Chazal and Gerlier, 2003) |
| | 39 | B0YLJ3 | 37.7 | 9.6 | 3 | | SP; Threonine-rich regions (123-197) interrupted by proline/serine residues (167-179) | Heavily *O*-glycosylated protein – cellular binding (McGeoch *et al.*, 1993) |
| | 36 | B0YLJ0 | 13.8 | 18.3 | 3 | ORF67, WSSV | SP, Thymidylate synthase | Protection of viral genome from host DNAses (Forterre *et al.*, 2004) |
| | 68 | B0YLM2 | 12.7 | 32.4 | 4 | | | |
| | 72 | B0YLM6 | 31.8 | 22.3 | 6 | | TM; ERV/ALR sulfhydryl oxidase domain (157-254) | Disulphide-bond formation during cytoplasmic virus assembly (Hakim *et al.*, 2011; Hakim and Fass, 2009; Senkevich *et al.*, 2000b; 2000a) |
| | 45* | B0YLJ9 | 201.1 | 10.2 | 15 | | PPASE-TENSIN (299-603); Coiled-coil region (533-542); *t*-SNAREs (466-528); SF3 Helicase (1411-1581); Asparagine-rich region (417-454) | Site-specific host-virus interactions and packaging (James *et al.*, 2003) |
| Nucleocapsid components | 62* | B0YLL6 | 512.1 | 9.1 | 30 | ORF147, *T. ni* ascovirus-2c | SP; NLS-BP (4357-4373), Nebulin-repeats (2541-2561 and 4207-4231); GBD-FH3 (856-1247; 1492-1885; 2138-2557); Leucine-zipper (1148-2093); ezrA (964-1584); NUMOD3 motifs (850-863; 3416-3429; 3465-3478); Spectrin repeats (976-1066; 1162-1299; 1546-1649); *t*-SNAREs (1677-1705); Ag332 (2804-2869); | Nuclear targeting (Robbins *et al.*, 1991); actin-zipper (Labeit and Kolmerer, 1995); homing endonuclease (Sitbon and Pietrokovski, 2003); Transcription regulation (Groves *et al.*, 2001); |

| Predicted Classification¥ | ORF No. | UniProt IDs | Mol. mass [kDa] | Coverage [%] | Peptides identified | Homology to cellular proteins and/or proteins in other viruses | Signature domain(s)/Motifs (amino acid regions harbouring the motifs/domains) | Functional annotation (References) |
|---|---|---|---|---|---|---|---|---|
| Nucleocapsid components | 83 | B0YLN7 | 81.6 | 16.6 | 9 | ORF AMV214 *A. moorei* EPV | NLS-BP | Nuclear targeting (Robbins *et al.*, 1991) |
| | 104 | B0YLQ8 | 77.9 | 7.6 | 4 | | TM, Coiled coil region | |
| | 108* | B0YLR2 | 63.9 | 19.4 | 8 | Cell-division protein 48, Lymphocystis disease virus (China isolate) | SP; AAA-ATPase Central domain protein (240 to 384); PAN (50 to 463); | Molecular chaperone/remodelling of macromolecules (Lyer *et al.*, 2004); DNA unwinding and/or packaging (Gorbalenya and Koonin, 1989) |
| | 107* | B0YLR1 | 59.6 | 35.9 | 16 | Cell-division protein 48, Lymphocystis disease virus (China isolate) | SP; P-loop /AA-ATPase Central domain protein (212 to 388); PAN (27-430); | Molecular chaperone/remodelling of macromolecules (Lyer *et al.*, 2004); DNA unwinding and/or packaging (Gorbalenya and Koonin, 1989) |
| | 61 | B0YLL5 | 57.4 | 22.5 | 8 | | | |
| | 106* | B0YLR0 | 55.1 | 29.2 | 9 | | Serine/Threonine/Glutamine-rich stretches (25-106); | Sequence-specific transcription activation (Askovic and Baumann, 1997; Biggin *et al.*, 1988) |
| | 70 | B0YLM4 | 50.9 | 21.3 | 5 | | | |
| | 44 | B0YLJ8 | 42.8 | 16.7 | 5 | | Glutamine-rich region (265-286) | |
| | 154 | B0YLV8 | 40.1 | 11.8 | 4 | | | |
| | 52 | B0YLK6 | 36.7 | 5.6 | 2 | Cellular protein, *P. falciparum* | | |
| | 31 | B0YLI5 | 33.6 | 16.8 | 5 | | Leucine/isoleucine-rich regions (4-30); *N*-myristoylation sites (48-53); HDAC-interaction like domain protein | Export of macromolecules from nucleus to cytoplasm (Gorlich and Kutay, 1999); Gene regulation (Gwack *et al.*, 2001; Kuo and Allis, 1998; Zhu *et al.*, 1999) |
| | 113* | B0YLR7 | 33.1 | 30.6 | 8 | Cellular protein PY00593, *P. y yoelli* Str.17XNL | SP | |
| | 98* | B0YLQ2 | 13.5 | 44.3 | 7 | | TM | |
| | 101* | B0YLQ5 | 12.3 | 46.2 | 6 | | TM, SP | |
| Tegument components | 38* | B0YLJ2 | 136.7 | 46.3 | 47 | | SP; RGD motif (914-916) | Virus-host cell interactions during viral entry (Bai *et al.*, 1993; Belin and Boulanger, 1993; Roivainen *et al.*, 1991; Ruoslahti, 1996; Shayakhmetov *et al.*, 2005) |
| | 10 | B0YLG4 | 127.0 | 68.4 | 81 | ORF MSV156, *M. sanguinipes* EPV | Potential *N*-glycosylation sites (157, 313, 327 and 342); multiple serine/threonine/tyrosine-rich regions; Proline-rich profile (292-357); Bipertite-NLS (232-249); Bromodomain-2 profile (662-732); PPASE-TENSIN (801-1013) | Nuclear targeting (Robbins *et al.*, 1991); recruitment of proteins in signalling (Kay *et al.*, 2000); chromatin regulation of virus infection (Lieberman, 2006; Nicewonger *et al.*, 2004); |
| | 71 | B0YLM5 | 72.0 | 4.1 | 2 | | | |
| | 86 | B0YLP0 | 70.2 | 43.8 | 25 | | | |
| | 64 | B0YLL8 | 70.0 | 47.4 | 29 | ORF AMV130 *A. moorei* EPV | ATP-binding cassette transporter; PUM (1-14) | Regulation of cholesterol efflux (Hakim and Fass, 2009); |

| Predicted Classification[¥] | ORF No. | UniProt IDs | Mol. mass [kDa] | Coverage [%] | Peptides identified | Homology to cellular proteins and/or proteins in other viruses | Signature domain(s)/Motifs (amino acid regions harbouring the motifs/domains) | Functional annotation (References) |
|---|---|---|---|---|---|---|---|---|
| Tegument components | 46 | B0YLK0 | 61.5 | 41.7 | 18 | | PPASE (inorganic pyrophosphatase) (438-444) | Recruitment of proteins in signalling (Kay *et al.*, 2000) |
| | 47 | B0YLK1 | 47.2 | 38.1 | 17 | Cellular protein (CBG22662); *C. briggsae* | | |
| | 97* | B0YLQ1 | 44.4 | 55.1 | 24 | | TM, SP | |
| | 96* | B0YLQ0 | 43.5 | 66.7 | 28 | | | |
| | 7 | B0YLG1 | 41.0 | 7.9 | 3 | | | |
| | 2 | B0YLF7 | 38.7 | 46.8 | 17 | | | |
| | 93* | B0YLP7 | 38.5 | 55 | 19 | | | |
| | 50 | B0YLK4 | 32.7 | 44.7 | 12 | | | |
| | 94 | B0YLP8 | 32.7 | 66.7 | 18 | | TM | |
| | 67* | B0YLM1 | 31.0 | 62.9 | 18 | | | |
| | 69* | B0YLM3 | 30.9 | 62.6 | 20 | | | |
| | 85 | B0YLN9 | 30.1 | 38.4 | 10 | | | |
| | 110 | B0YLR4 | 23.6 | 11.4 | 2 | Matrixin peptidase, *S. litura* GV | TM, SP, Zinc-dependent metalloprotease | Involved in ectodomain shedding (Dolnik *et al.*, 2004) |
| | 112 | B0YLR6 | 19.1 | 24 | 6 | | TM, SP | |
| | 43 | B0YLJ7 | 16.9 | 61.1 | 9 | | TM | |

[¥] it should be noted that the classification used here is predictive (see text for discussions); more investigations are required to validate these predictions.

SP, signal peptide; TM, trans-membrane domain; RGD, Arg-Gly-Asp/cell attachment sequence; HDAC, Histone deacetylase; SCG, serine-cystein-glycine; NLS, nuclear localization signal; GBD-FH3, Rho GTPase-binding/formin homology 3 domain; ezrA, septation ring formation regulator ezrA; NUMOD3, nuclease-associated modular DNA-binding domain 3; PPASE-TENSIN, pyrophosphatase tensin-type domain profile: PUM, Pumilio RNA-binding repeat profile.

Motif analyses using ExPASy revealed several key features in virion protein sequences, among which are an arginine-glycine-aspartate (RGD) motif/cell attachment sequence (ORF38), bipartite nuclear localization signals (NLS-BP) (ORFs 10, 62 and 83) and a P-loop nucleotide-binding motif (ATP/GTP-A2) (ORF107). In some viruses such as adenoviruses, RGD domains have been shown to be involved in virus-host interactions during internalization and egress of virus particles (Bai *et al.*, 1993; Roivainen *et al.*, 1991; Shayakhmetov *et al.*, 2005). NLS-BP has been shown to be involved in targeting of viruses to the nucleus after entry into host cell (Robbins *et al.*, 1991). Some GpSGHV proteins have regions enriched in specific amino acids such as proline (ORFs 10 and 39), isoleucine/leucine, serine, threonine, glutamine, asparagine, glutamine (See **Table 2** for details). Such amino acid-enriched regions have been reported to play important roles: for instance, proline-enriched proteins have signalling roles (Kay *et al.*, 2000). Fourteen of the identified GpSGHV proteins have homologs in other viruses, while four showed homology to known cellular proteins (**Table 2**). Analysis of the phosphorylation status of the GpSGHV proteins by Western blot showed six major signals in the intact virus sample (marked with asterisks in **Fig. 7**; two signals of 170 kDa, and signals of ~43, 38, 30 and 15 kDa.

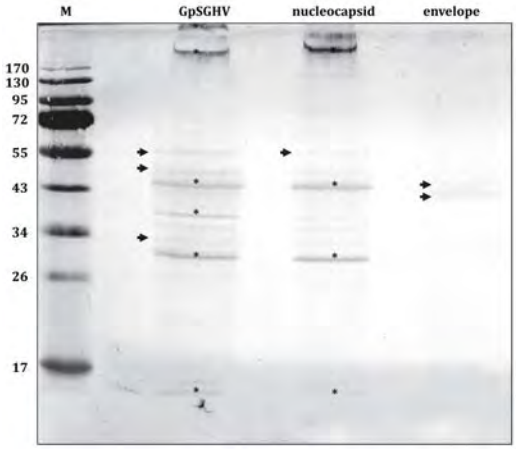


**Figure 7: Analysis of the phosphorylation of GpSGHV proteins by Western Blot:** Western blot analysis of the phosphorylation statuses of GpSGHV structural proteins in intact virions, nucleocapsid and envelope fractions samples. Major phosphorylated proteins in intact virus sample and nucleocapsid fraction are indicated by asterisks (*), minor proteins are indicated by arrowheads.

Four signals were also visualized in the nucleocapsid fraction. Several minor bands were observed in the intact virion preparation, some of which were also observed in the nucleocapsid fraction. Only two signals were observed in the envelope fraction ($M_r$ of ~44 kDa). Bioinformatics analysis of the viral proteins in the structural components indicates that at least six proteins (encoded by ORFs 38, 67, 69, 93, 96, 97) are likely to be phosphorylated. Based on the molecular sizes of the proteins identified by LC-MS/MS, the phosphorylation of these proteins was confirmed by the Western blot analysis (marked with asterisks in **Table 2**; see also **Fig. 7**). In addition, based on the distribution of the GpSGHV detected by LC-MS/MS and bioinformatics tools, the

majority of the phosphorylated viral proteins could be predicted to be localized in the viral tegument. However, it should be noted that this is just prediction: more investigations that are experimental are required to validate whether this is the case, for instance by using immunocytochemistry on thin sections. Whereas no common motifs have been identified to direct proteins into the viral tegument, it has been suggested for herpes viruses that phosphorylation facilitates incorporation of proteins into the viral tegument (Kalejta, 2008), with the majority of tegument proteins being phosphorylated (Shenk and Stinski, 2008). Viral phospho-proteins may have significant influence on the assembly (Sen *et al.*, 1977; Sen and Todaro, 1977), un-coating of virions (Lackmann *et al.*, 1987; Witt *et al.*, 1981), the interaction between viral proteins and host DNA (Scheidtmann *et al.*, 1984), and transcriptional regulation (Hsu *et al.*, 1982; 1985; Kingsford and Emerson, 1980).

### *Host cell-derived proteins in GpSGHV virions*

During morphogenesis, enveloped dsDNA viruses acquire host cell-derived proteins as integral part of the mature virions, especially the envelopes. These host-derived proteins may influence viral morphogenesis; virus-host cell interactions, especially host responses to virus-incorporated self-proteins and induction of pathogenesis. In this study, 51 host cell-derived proteins were identified in the GpSGHV virion proteome. Of these, eight were measurable only in the nucleocapsid fraction, including several 26S/60S ribosomal proteins, histone H3-II, phage terminase (*Sodalis glossinidius*; strain 'morsitans'), and vesicle coat complex COPI-ε (**Table 3** and **Fig. 5**). Similarly, five of the cellular proteins were measurable only in the envelope fraction, including cargo transport protein EMp24, major outer membrane lipoprotein (*Sodalis glossinidius*; strain 'morsitans'), F0F1-type ATP synthase-β and an uncharacterized membrane trafficking protein. Other cellular proteins were detected in both the envelope and nucleocapsid fractions with varying abundances (see **Fig. 5**). Enzymatic codes could be assigned to twenty-two of the fifty-one cellular proteins.

**Table 3: Cellular proteins identified in purified GpSGHV virions by LC-MS/MS:** Enzyme code mapping is shown for relevant proteins in column 2. Host cellular molecules acquired and incorporated by some enveloped viruses are marked with asterisk.

| Classification | Host molecule [EC mapping] | UniProt Protein ID | Peptides identified | Coverage (%) | Functional annotations | Other viruses in which association has been identified |
|---|---|---|---|---|---|---|
| Protein Biosynthesis | Eukaryotic translation elongation factor 2 | D3TQG6 | 15 | 42.7 | Translation elongation activity | HIV-1 (Chertova *et al.*, 2006), HCMV (Varnum *et al.*, 2004), KSHV (Zhu *et al.*, 2005), SARS-CoV (Neuman *et al.*, 2008) |
| | 26S proteasome regulatory complex ATPase RPT1 (Subunit 7) [EC:3.6.1.3] | D3TS21 | 6 | 18.8 | Anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process | SARS-CoV (Wang *et al.*, 2010) |
| | Aspartyl tRNA synthetase * [EC:6.1.1.12] | D3TLE4 | 5 | 10.0 | Protein biosynthesis/ligase | HIV-1 (Halwani *et al.*, 2004), HIV-1 (Halwani *et al.*, 2004) |
| | 40S Ribosomal proteins § (S2; S3A; S6; S7; S8; S16; S20; S24; S27a) | D3TQI9; D3TQN0; D3TLR2; D3TR24; D3TL24; D3TS86; D3TMY7; D3TPY6; D3TSD5; D3TMY6 | 4; 12; 2; 7; 7;6; 3; 2; 2; 4 | 15.9; 43.7; 8.5; 36.8; 36.2; ; 39.7; 28.3; 16.7; 21.2; 56.3 | Structural constituent of ribosome (cytosolic small ribosomal subunit); Protein synthesis and modification | VEE (Montgomery *et al.*, 2006), Filoviruses EBOV and MARV (Spurgers *et al.*, 2010) |
| | 60S Ribosomal proteins § (L6; L7; L9; L10; L10a; L11; L13; L13a; L14; L18a; L21; L23; L31; L34) | D3TMG2; D3TLP1; D3TR48; D3TLP4 ; D3TMC2; D3TS85; D3TPE1; D3TSE5; D3TQU5; D3TQX4; D3TKZ4; D3TMN9; D3TKJ4; D3TQW9 | 5; 4; 4; 8; 4; 3; 8; 5; 5; 6; 4; 3; 5; 2 | 18.2; 12.9; 19.5; 37.0; 20.3; 15.3; 30.7; 17.6; 30.1; 30.5; 32.1; 12.5; 41.1; 13.1 | Structural constituent of ribosome (mitochondrial and cytosolic large ribosomal subunit); | |
| | Translation elongation factor EF-1α | D3TPG3 | 7 | 12.8 | Protein synthesis | HIV-1 (Chertova *et al.*, 2006; Cimarelli and Luban, 1999; Ott *et al.*, 2000b; Saphire *et al.*, 2006), VV (Chung *et al.*, 2006; Resch *et al.*, 2007), MCMV (Kattenhorn *et al.*, 2004), HCMV (Varnum *et al.*, 2004), SARS-CoV (Neuman *et al.*, 2008), HIV-1 (Chertova *et al.*, 2006; Ott *et al.*, 1998; Saphire *et al.*, 2006), SIV (Ott *et al.*, 1998), MMLV (Ott *et al.*, 1998; Segura *et al.*, 2008), (Chung *et al.*, 2006; Webb *et al.*, 1999) |
| | Eukaryotic initiation factor-5C | D3TLI4 | 3 | 8.1 | DEAD box ATP-dependent RNA helicase; mRNA splicing; Regulation of mRNA translation | LCMV (Campbell Dwyer *et al.*, 2000), HIV-1 (Cimarelli and Luban, 1999) |
| | Eukaryotic translation initiation factor 3 (sub units-G; 6 and 2β) | D3TLX5; D3TM41 and D3TRV5 | 2; 6; and 2 | 10.4; 14.7 and 8.6 | Protein synthesis | |
| | Serine/Threonine-protein phosphatase PP1 | D3TNN2 | 7 | 26.8 | Regulation of protein synthesis | HCMV (Michelson *et al.*, 1996) |
| | PolyA-binding protein | D3TQ38 | 8 | 15.3 | RRM dimerization, RNA/DNA binding, PABP | See detailed review in (Walsh and Mohr, 2011) |
| Cytoskeleton | Tubulin (β-1 chain, α-subunit) * | D3TR30; Q9BMF4 | 19; 10 | 49.7; 45.0 | Microtubule-based movement | Influenza (Shaw *et al.*, 2008), HCMV (Varnum *et al.*, 2004), VV (Chung *et al.*, 2006; Resch *et al.*, 2007), HIV-1 (Chertova *et al.*, 2006), ASFV (Esteves *et al.*, 1986), EBV (Johannsen *et al.*, 2004), (Segura *et al.*, 2008), EBOV (Spurgers *et al.*, 2010), MMLV (Wang *et al.*, 2003) |

| Classification | Host molecule [EC mapping] | UniProt Protein ID | Peptides identified | Coverage (%) | Functional annotations | Other viruses in which association has been identified |
|---|---|---|---|---|---|---|
| | Actin* (5C; 87E [EC:2.4.1.17]) | D3TQK0; D3TPT6 | 21; 23 | 55.3; 73.1 | Cytokinesis; Positive regulation of NFAT protein import into the nucleus | Influenza (Shaw *et al.*, 2008), HCMV (Varnum *et al.*, 2004), KSV (Zhu *et al.*, 2005), AIHV-1 (Dry *et al.*, 2008), SeV (Lamb, 1975), MeV (Tyrrell and Norrby, 1978), RV (Naito and Matsumoto, 1978), MMLV (Wang *et al.*, 2003) |
| | Troponin | D3TS62 | 10 | 24.4 | Actin-binding | HIV-1 (Tomasselli *et al.*, 1991) |
| | Myosin (heavy chain) | D3TQ00 | 70 | 67.0 | Microfilament motor activity | VV (Chung *et al.*, 2006), KSHV (Zhu *et al.*, 2005) |
| | Filamin- α | D3TSC9 | 7 | 8.9 | Actin-binding protein | HIV-1 (Cooper *et al.*, 2011) |
| Fatty acid metabolism | ADH-II [EC:1.2.1.3; EC:1.2.1.10] | D3TS11 | 2 | 4.0 | Fatty acid metabolism | |
| | Medium-chain acyl-CoA dehydrogenase [EC:1.3.99.3] | D3TNV6 | 6 | 18.6 | Metabolism of carnitine (required for transport of fatty acids into mitochondria) | |
| | Prohibitin-like protein | D3TLB8 | 10 | 47.1 | Lipid raft-associated integral membrane protein | HIV-1 (Emerson *et al.*, 2010) |
| | Hydroxyacyl-CoA hydratase [EC:4.2.1.74; EC:1.1.1.35; EC:1.1.1.211; EC:4.2.1.17] | D3TSD9 | 10 | 14.1 | Crotonase | HCV (Diamond *et al.*, 2010) |
| Ion transport | F0F1-type ATP synthase (β-subunit) [EC:3.6.3.6] | D3TMR0 | 4 | 25.4 | Proton-transporting ATPase | HIV-1 (Chertova *et al.*, 2006), VV (Jensen *et al.*, 1996), EBOV (Spurgers *et al.*, 2010) |
| | Mitochondrial F1F0-ATP synthase (ε-subunit) [EC:3.6.3.6] | D3TS81 | 2 | 21.5 | Proton-transport | TMEV (Sorgeloos *et al.*, 2011) |
| | Porin | D3TRY2 | 7 | 30.1 | Voltage-gated anion channel activity | Bacteriophage Sf6 (Zhao *et al.*, 2011) |
| Signal transduction | Endocytosis/signalling protein (EHD1) [EC:3.1.3.2] | D3TMZ1 | 5 | 9.9 | Molecular chaperoning/signalling | |
| | GTPase Ran/TC4/Gsp1 | D3TM84 | 7 | 35.3 | Nucleocytoplasmic transport | HSV-1 (Zenner *et al.*, 2011) |
| Cellular metabolism | GAPD* [EC:1.2.1.12] | D3TRU0 | 8 | 33.6 | Glycolysis | MCMV (Kattenhorn *et al.*, 2004), HIV-1 (Ott *et al.*, 2000a) |
| | Metalloexopeptidases [EC:3.4.13.0; EC:3.4.13.0] | D3TRW2 | 5 | 15.5 | Arginine biosynthesis | |
| | Pyruvate carboxylase [EC:6.4.1.1; EC:6.3.4.14] | Q0QHL4 | 10 | 29.4 | Gluconeogenesis | |
| | Succinyl-CoA synthetase (β) [EC:6.2.1.5] | D3TM34 | 7 | 16.2 | Succinate-CoA ligase (ADP-forming) activity | |
| | Peroxiredoxin | D3TNB1 | 8 | 35.0 | Glutathione peroxidase | MeV (Watanabe *et al.*, 2011) |
| | Acyl-CoA reductase [EC:1.2.1.0] | D3TP92 | 5 | 12.2 | Oxidoreductase using NAD/P as cofactor | HCV (Diamond *et al.*, 2010) |
| | Arginine kinase [EC:2.7.3.3] | D3TPN5 | 13 | 49.2 | Maintenance of cell homeostasis | HIV-1 (Hui, 2002) |
| | Fructose-bisphosphate aldolase [EC:2.7.11.0; EC:4.1.2.13] | D3TPV3 | 10 | 43.6 | Protein serine/threonine kinase activity | |
| | γ -glutamyl phosphate reductase ]EC:2.7.2.11; EC:1.2.1.41] | D3TQ43 | 17 | 24.4 | Glutamate 5-kinase activity | |
| | Protein disulfide isomerase [EC:5.3.4.1] | D3TLX2 | 8 | 17.8 | ER Thioredoxin | |
| Protein processing | Heat shock proteins § (p23*; Hsp70-cognate-3 [EC:3.6.1.3]; -4 | D3TMP8; D3TRH2; D3TPL1; D3TRQ0; | 2; 23; 28; 5; 11 | 15.9; 38.8; 50.2; 9.4; 21.4 | Protein-protein interactions | HIV-1 (Gurer *et al.*, 2002), Hepadnavirus (Hu *et al.*, 1997) |

| Classification | Host molecule [EC mapping] | UniProt Protein ID | Peptides identified | Coverage (%) | Functional annotations | Other viruses in which association has been identified |
|---|---|---|---|---|---|---|
| machinery | [EC:2.7.11.0; EC:3.6.1.3] and -5 [EC:3.6.1.3; EC:1.3.1.74]); Hsp 60 (GroEL) Chaperonin complex component TcP-1 subunit-γ | D3TR05 D3TN28 | 20 | 37.4 | Protein folding; cytoplasmic microtubule organization | M-PMV (Hong *et al.*, 2001) |
| | Molecular chaperone | D3TLT0 | 8 | 26.0 | Unfolded protein binding | EBV (Young *et al.*, 2008) |
| | FKBP-type peptidyl-prolyl cis-trans isomerase * [EC:5.2.1.8] | D3TLE5 | 2 | 6.1 | Protein degradation and sorting | HIV-1 (Briggs *et al.*, 1999; Dorfman and Gottlinger, 1996), |
| | Ubiquitin */40S – (S27a fusion protein) | D3TMY6 | 4 | 56.3 | Viral reproduction | AcNPV (Guarino *et al.*, 1995), ASFV (Webb *et al.*, 1999), Influenza (Shaw *et al.*, 2008), HIV-1 (Garrus *et al.*, 2001), SIV (Ott *et al.*, 1998), |
| | Ubiquinol cytochrome C reductase [EC:1.10.2.2] | D3TPJ2 | 5 | 14.7 | Cleavage of leader peptides in nascent proteins | |
| Transcription regulation | Histone H2A | D3TPW0 | 5 | 34.8 | Nucleosome assembly | HSV-1 (Cliffe and Knipe, 2008; Lieberman, 2006);MCMV (Kattenhorn *et al.*, 2004), Simian virus 40 (Chen *et al.*, 1979) |
| | Putative H3 histone family 2 (Isoform II) | D3TKL3 | 4 | 19.9 | Regulation of gene silencing via chromatin-binding | HSV-1 (Cliffe and Knipe, 2008; Lieberman, 2006) |
| | Phage terminase (*Sodalis glossinidius*) | Q2NTN5 | 2 | 16.7 | DNA packaging | Bacteriophage SaPIs (Tallent *et al.*, 2007) |
| Protein Export | Rab proteins (1;10) | D3TLV4; D3TNT5 | 4; 4 | 19.0; 16.7 | ER to Golgi vesicle-mediated transport | HSV-1 (Zenner *et al.*, 2011), EBOV (Spurgers *et al.*, 2010), CaMV (Leh *et al.*, 2000) |
| | Vesicle coat complex COPI- ε | D3TM22 | 4 | 24.9 | Retrograde vesicle-mediated transport, Golgi to ER | MNV-1 (Hyde *et al.*, 2009; Yoshizuka *et al.*, 2005) |
| | Putative cargo transport protein EMp24 | D3TM93 | 4 | 20.3 | Cellular transport | VV (Husain and Moss, 2003) |
| | Membrane trafficking protein | D3TQM6 | 5 | 26.4 | Golgi organization | |
| | ADP/ATP translocase | D3TNQ0 | 16 | 47.7 | ADP antiporter | |
| Endosymbiont proteins | Outer membrane protein (OmpA/F) (*Sodalis*) | Q2NU70; Q2NU93 | 10; 4 | 29.5; 13.3 | Integral membrane proteins (pore complex) with porin and iron transport activity | |
| | Major outer membrane lipoprotein (*Sodalis*) | Q2NT13 | 5 | 61.5 | Maintenance of structural and functional integrity of cell envelope | MIMI (Renesto *et al.*, 2006), |
| | Hypothetical phage protein (*Sodalis*) | Q2NU11 | 2 | 50.9 | - | |
| | Peptidoglycan-associated lipoprotein (Pal) (*Sodalis*) | Q2NUL3 | 2 | 11.8 | A ubiquitous Toll-like receptor-2 agonist | |
| | Type III secretion apparatus (*Sodalis*) | Q2NVI4 | 10 | 10.7 | Membrane-associated protein | |
| | WIGBR Periplasmic folding factor D (PpiD protein) (*Wigglesworthis*) | Q8D349 | 2 | 2 | Maturation of the major β-barrel outer membrane proteins (OMPs) | |

§ For these proteins, the protein IDs (column 3), No. of identied peptides (column 4) and the percent coverage (column 5) are shown in the same order of listing in column 2.

**Table 4: Verification of incorporation of cellular proteins in the mature GpSGHV virion:** Purified virions were either treated with 0.08 µg of proteinase K (protK) per one µg of virion protein or treated and purified through a 20% Ficoll cushion followed by LC-MS/MS analysis.

| Classification | Host molecule | UniProt IDs | Mass [kDa] | Unique peptides | Incorporated (in other viruses) | Virus (Reference) |
|---|---|---|---|---|---|---|
| Protein Synthesis | Elongation factor 1-α | D3TNV8 | 50.4 | 6 | No | HIV-1 (Cimarelli and Luban, 1999), WNV (Blackwell and Brinton, 1997) |
| | Elongation factor 2 | D3TP87 | 94.5 | 5 | | |
| Protein processing machinery | Hsc70-4 | D3TPL1 | 60.8 | 4 | Yes | HIV-1 (Gurer *et al.*, 2002) |
| | Hsc70-3 | D3TRH2 | 72.6 | 10 | | |
| Transcription regulation | Histone H2A | D3TPW0 | 15.0 | 2 | Yes | SV-40 (Chen *et al.*, 1979) |
| Cytoskeleton | Myosin (heavy chain) | D3TQ00 | 87.3 | 30 | Yes | HIV-1 (Ott *et al.*, 1996; Ott *et al.*, 2000b) |
| | Tubulin-α | D3TQG7 | 49.9 | 11 | Yes | Mo-MuLV (Wang *et al.*, 2003) |
| | Actin 5C | D3TQK0 | 41.8 | 3 | Yes | Mo-MuLV (Nermut *et al.*, 1999; Wang *et al.*, 2003), |
| | Tubulin-β | D3TR30 | 50.2 | 7 | | |
| Cellular metabolism | Glyceraldehyde-3-phosphate dehydrogenase | D3TRU0 | 35.7 | 2 | Yes | HIV-1 (Ott *et al.*, 2000a); |
| | ER glucose-regulated protein | D3TS03 | 82.1 | 16 | | |
| | Protein disulfide isomerase | D3TRE3 | 57.4 | 3 | No | HIV-1 (Ott, 2002) |
| Ion transport | Porin | D3TRY2 | 30.4 | 5 | | |

Treatment of purified GpSGHV particles with protK removed many of the proteins associated with the viral particle (**Fig. 8 a)**. After passing the protK-treated sample through a 20% Ficoll cushion followed by LC-MS/MS, none of the ten virion envelope proteins were detectable in contrast to the majority of the tegument proteins. Based on the molecular weights, the major viral proteins that were undetectable after protK-treatment (marked with asterisks in **Fig. 8 a, lane 2**) include proteins encoded by ORF45 (201 kDa), ORF38 (137 kDa), ORF107 (60 kDa), ORFs 47 and 97 (~ 50 kDa), ORFs 69 and 85 (~ 30 kDa), ORFs 68 and 101 (~ 12 kDa). When observed under TEM, the ProtK-treated virus particles were devoid of intact envelopes (**Fig. 8 c**; compare with **Fig. 8 b**). Thirteen host proteins were still identified in the sample that was passed through the 20% Ficoll cushion. Of these proteins, six (heat shock cognate-70 family proteins, histone H2A, myosin, tubulin, actin and glyceraldehyde-3-phosphate dehydrogenase) have been demonstrated to be incorporated in other viruses (**Table 4** and the references therein). Western blot analysis of the protK-treated samples with antibodies against cellular host proteins produced a clear signal with the anti-myosin IgG (**Fig. 8 d**). Western blot analysis using antibodies against the other tested cellular host proteins showed either a negative signal (actin) or a weak signal (ubiquitin and tubulin). Limitations of virus quantities, however, precluded the optimization of the protocol for detection of these host proteins.

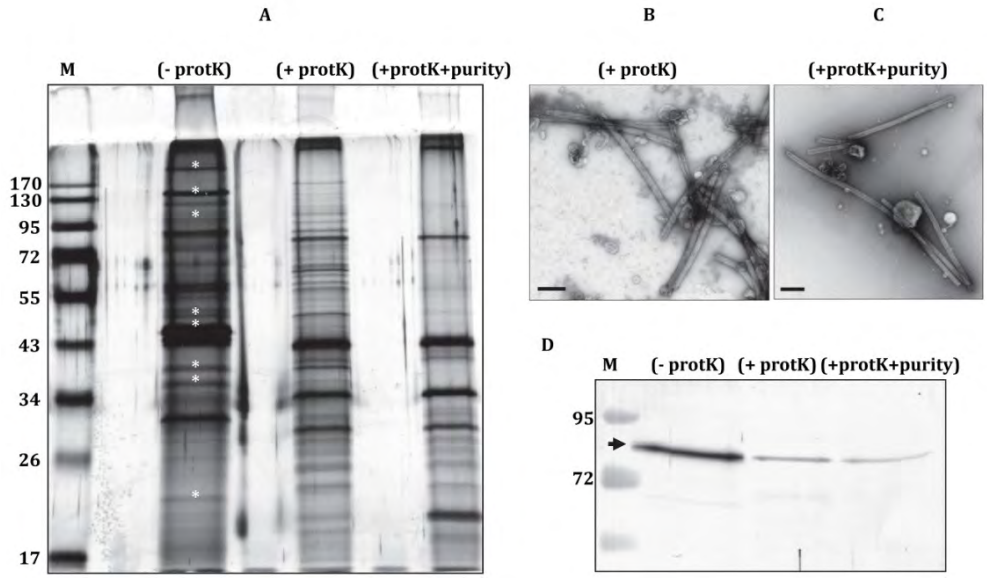


**Figure 8: Proteinase K protection assay of purified GpSGHV virions:** (A) Silver-stained SDS-PAGE gel of non-treated (-protK), ProtK-treated (+protK) and ProtK-treated sample which was passed through a 20% Ficoll cushion to remove protK and free-floating peptides (+protK+purity). ProtK-treatment followed by 20% Ficoll-purification resulted to naked nucleocapsids (Compare panel (B) and (C)). Western blot with rabbit polyclonal anti-myosin (MYH) antibody detected myosin in the (+protK+purity) fraction (D) Viral proteins that disappeared after ProtK-treatment are indicated by asterisks in Lane 2. Bars represent 200 nm (B) and (C).

## *Implications of finding host proteins in GpSGHV*

These studies clearly show that GpSGHV virions contain numerous cellular proteins. Cellular host proteins incorporated into or binding onto virus particles have been demonstrated to play specific or supplementary roles in the virus 'life-cycles'. Actin and myosin have been reported to be incorporated, for instance, into Moloney murine leukemia virus (Mo-MuLV) (Nermut *et al.*, 1999) and in human immunodeficiency virus type 1 (HIV-1) (Ott *et al.*, 1996; Ott *et al.*, 2000b). In HIV-1, inhibition of the interaction between the Gag protein with actin and myosin markedly reduced the amount of virus released from infected cells (Ott, 1997). Glyceraladehyde-3-phosphate dehydrogenase (GADPH) has been reported to be co-incorporated with actin inside mature HIV-1 virions, where it plays roles in enhancement of gene expression (Ott *et al.*, 2000a). Translation elongation factor-1 alpha (EF-1α) has been proposed to target West Nile virus (WNV) RNA to a microenvironment for efficient viral replication (Blackwell and Brinton, 1997), and plays roles in packaging of HIV-1 into nascent virions (Cimarelli and Luban, 1999). Heat shock protein 70 family members have been demonstrated to be bona fide proteins of primate lentiviral

virions, and have been proposed to play roles in virus assembly and egress (Gurer *et al.*, 2002). Finally, virions of Simian Virus 40 (SV40) have been demonstrated to contain the biosynthetically active histone H2A protein (Chen *et al.*, 1979). Taken together, it seems likely that the cellular host proteins in the GpSGHV virions may have specific or auxiliary roles in the virus 'life-cycle'. Further investigations are needed to find out whether the incorporated cellular proteins are distributed over infectious and non-infectious GpSGHV virions.

## Conclusions

The GpSGHV virion has a rod-shaped protein nucleocapsid core surrounded by a proteinaceous tegument, an outer envelope and helical surface projections. The predicted tegument proteins of GpSGHV comprise almost 50% ($n$ = 20) of the total virion proteins ($n$=61). GpSGHV contains numerous virion-associated cellular proteins, some of which appear to be specifically incorporated into the mature virion. Potentially, the presence of cellular-derived proteins in GpSGHV virions reflects the requirement of these proteins during GpSGHV infection process. Alternatively, the cellular-derived proteins may be remnants of GpSGHV-host interactions. The GpSGHV progeny nucleocapsids translocate to the cytoplasm where the entire envelopment appears to be orchestrated. Further experiments are required to investigate the precise mechanism and pathway by which GpSGHV acquires the envelop, for instance by analysing the lipidome of the virus. The cytoplasmic assembly of GpSGHV particles induces cellular damage, which culminates into disintegration of the cell plasma membrane as the mature virions egress from the infected cell. Finally, the data presented in this study offer new directions in antiviral strategies based on GpSGHV-host interactions. Potential strategies have been reviewed (Kariithi *et al.*, 2013c).

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# Chapter 5

# Salivary secretome GpSGHV-infected *G. pallidipes*[4]


## Abstract

The competency of the tsetse fly *Glossina pallidipes* (Diptera: Glossinidae) to support transmission of *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV; family *Hytrosaviridae*) may depend on complex virus-host interactions. Critical requisites to GpSGHV transmission are the viral replication and the secretion of viral particles and/or viral-encoded proteins into the fly's salivary gland lumen. Secretion of host proteins is of equal importance for successful virus transmission. This calls for cataloguing of the host's salivary gland proteins. This chapter describes the salivary gland secretome of asymptomatic and symptomatic laboratory-bred *G. pallidipes* and the effects of GpSGHV infection in the expression of host salivary gland proteins. Saliva was harvested and the proteins profiled by 12% SDS-PAGE. The separated proteins were excised from the gels and subjected to in-gel trypsin digestion, followed by protein identification using LC-MS/MS. The proteins were identified by MaxQuant/Andromeda analyses of the MS data against a non-redundant, and a tsetse fly salivary gland ESTs databases. Taking into account an FDR limit of < 1%, and a detection threshold of at least two unique peptides per protein, two hundred ninety-two host and twenty-five GpSGHV proteins were identified. When annotated by the Blast2GO suite, at least one GO term could be assigned to 89.9% (285/317) of the detected proteins. Five (~1.8%) host and three (~12%) GpSGHV proteins remained without any predicted function after blast searches against the databases. Sixty-five of the two hundred ninety-two detected host proteins contained N-terminal signal peptide sequences. Twenty-one of the twenty-five viral proteins showed potential interactions with several host secretome proteins.


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# Introduction

The tsetse fly *Glossina pallidipes* (Diptera: Glossinidae) is an important vector of African trypanosomosis in man and cattle, and is considered as one of the "roots of hunger and poverty" in sub-Saharan Africa (Vreysen, 2006). An effective method to control trypanosomiasis is the eradication of the vector (tsetse fly) by the sterile insect technique (SIT) as a component of area-wide integrated pest management (AW-IPM) (Vreysen, 2006). SIT requires production of sterile males in large-scale insect colonies. However, colonized *G. pallidipes* flies are infected by the *G. pallidipes* salivary gland hypertrophy virus (GpSGHV: family *Hytrosaviridae*), a double-stranded DNA (dsDNA) virus that can cause the collapse *G. pallidipes* colonies (Abd-Alla *et al.*, 2010b). In these colonies, the GpSGHV transmission is amplified by the *in vitro* membrane system, which is routinely used to feed the flies (Abd-Alla *et al.*, 2011b). Viremic flies release large amounts of virus particles via salivary secretions into the blood at each feeding event, and the released virions are infectious *per os* to healthy flies (**chapter 2**).

Potentially, the GpSGHV transmission in *G. pallidipes* colonies depends on complex virus-host interactions. There is evidence that some tsetse parasites such as trypanosomes modulates tsetse salivary protein content, thereby altering the fly's feeding behaviour and the parasites transmission dynamics (Champagne, 1994; van den Abbeele *et al.*, 2010). Despite the critical roles played by tsetse fly saliva during feeding, it is unknown whether GpSGHV modulates protein expression patterns. Given that knowledge on the mechanisms behind GpSGHV infection process remains limited, further studies are required to characterize the molecular interactions between the host and the virus. In this chapter, the salivary gland secretome of *G. pallidipes* was characterized through a parallel analyses of proteins harvested from asymptomatic and symptomatic flies. The aim of this study was to gain further insights into the fly's salivary gland secretome proteins, particularly in the identification of host-virus interacting protein partners.

# Materials and methods

## *Tsetse fly selection and handling*

Flies from a laboratory-bred *G. pallidipes* colony maintained at the IPCL Seibersdorf laboratories, Austria, were used in this study. Asymptomatic teneral flies (within 24 h post-eclosion) were screened by polymerase chain reaction (PCR) for GpSGHV infection. The PCR primers and amplification conditions that were used have been previously described (Abd-Alla *et al.*, 2007a; 2011a). Teneral and 10-day old symptomatic male and female flies were selected from the colony based on their external morphology as previously (see **chapter 1**). Occurrence of the salivary gland

hypertrophy syndrome (SGH) in the symptomatic flies was confirmed during subsequent dissections of the salivary glands (see below). Experimental flies were subsequently offered one blood meal and divided into eight groups (25 flies in each group) based on hours post feeding (hpf). Only flies that were fully engorged at the end of the 15-min feeding event were used in subsequent analyses. The fly groups were as follows: groups 1A and B at 0 hpf (non-fed = teneral, asymptomatic or symptomatic), and groups of the ten-day old flies at 48 (groups 2A and B), 72 (groups 3A and B) and 96 (groups 4A and B) hpf. Flies in groups 1A and B were dissected immediately after PCR results. After feeding, the flies in groups 2, 3 and 4 were maintained in the insectaria in standard rearing conditions (Feldmann *et al.*, 1992) until saliva harvesting at the respective hpf.

## *Harvesting of tsetse fly saliva*

Saliva was harvested using a method described by van den Abbeele *et al.*, (2007), with slight modifications. Briefly, the flies were first subjected to a cold-shock on ice (5-10 min; 4˚C), and 25 and 100 pairs of intact salivary glands were dissected from GpSGHV-infected (SGH-positive) and healthy (asymptomatic) fly groups, respectively. It should be noted that more asymptomatic than symptomatic flies were used here due to the small sizes and difficulties to dissect asymptomatic flies. The dissected glands were collected in 500 µl ice-cold, sterile PBS (pH 7.4) supplemented with EDTA-free protease inhibitor cocktail (ROCHE, Germany). Saliva fluid was allowed to diffuse out of the glands into the buffer for 2.5 h (on ice), followed by a brief centrifugation (500 rpm; 2 min; 4˚C). The supernatants, presumably containing the saliva (diffusate from intact glands), were filtered (0.45 µm filter), and immediately frozen in 100 µl aliquots at -80˚C until further analyses.

## *SDS-PAGE and LC-MS/MS*

Saliva proteins were thawed on ice and precipitated using four volumes of ice-cold acetone per volume of protein. Protein precipitation was allowed to proceed overnight at -20°C and precipitates pelleted by centrifugation. The pelleted proteins were dissolved in 50 mM Tris-Cl, pH 8.0, 5 mM TE buffer (pH 8.0) containing 0.5% SDS and the total protein amounts present in the samples were estimated by the BCA protein assay according to standard protocols. For loading the protein samples on SDS-PAGE (12%) gels, samples containing equal amounts of total proteins were supplemented with SDS-sample buffer, boiled for 5 min at 95°C, and after electrophoresis (for 1.1 cm), the gels were stained with CBB - Colloidal Staining Kit (Invitrogen). The middle sections spanning entire gel lanes were excised, excluding flanking regions to avoid contaminations from the neighbouring gel lanes. In-gel trypsin digestion of gel pieces and analyses of resultant tryptic peptides by LC-MS/MS were performed as described in **Chapter 4** (Kariithi *et al.*, 2010).

### *Identification of saliva proteins*

Identification of saliva proteins was performed as previously described (Kariithi *et al.*, 2010). Briefly, raw MS/MS data were analysed using the MaxQuant software version 1.1.1.36, supported by Andromeda as the database search engine for peptide/protein identification (Cox *et al.*, 2011; Cox and Mann, 2008). MS/MS spectra were searched against a concatenated *G. m. morsitans* database. The database used for peptide/protein searches was derived from the salivary glands expressed sequence tag (EST) library (http://old.genedb.org/). Protein sequences of common contaminants, e.g. trypsin and keratins were used in MaxQuant's contaminants database. MaxQuant was used with a peptide tolerance of 10 parts per million (ppm) while all other settings were kept as default with one extra addition of N or Q de-amidation as variable modification (Hubner and Mann, 2011) to allow quantification of de-amidated peptides. Bioinformatics analyses of the MaxQuant/Andromeda workflow output and the analysis of the abundances of the identified proteins were performed with the Perseus module (available at the MaxQuant suite). Only peptides and proteins with a false discovery rate (FDR) of less than 1% and proteins with at least two unique peptides were accepted for further analyses.

### *Structural and functional annotation of saliva proteins*

The identified proteins sequences were inputted into Blast2GO v.2.4.8 (Conesa *et al.*, 2005), and categorized by molecular function, biological process and cellular component. Gene Ontology (GO) term mapping was based on sequence similarity to mapped UniProt database sequences, and by merging GOs obtained from InterProScan searches (http://www.ebi.ac.uk/Tools/pfa/iprscan/). Signal peptides were predicted from InterProScan searches (Zdobnov and Apweiler, 2001), while potential secretion signal peptide sequences were predicted by SignalP v. 3.0. (http://www.cbs.dtu.dk/services/SignalP/). Structural and functional annotations were determined by pasting the single-letter amino acid codes of the proteins into the Sequence Annotated Structure (SAS) interface (http://www.ebi.ac.uk/thornton-srv/databases/sas/) (Milburn *et al.*, 1998). Further annotations obtained from the PDB output were performed at the pfam site (http://pfam.sanger.ac.uk/).

## Results

### *Experimental strategy*

Analyses of secretome proteins was performed by: (1) electrophoretic profiling and identification of tsetse saliva proteins by LC-MS/MS, (2) cataloguing of the MS-supported proteins by GO mapping using Blas2GO suite; (3) confirmation of the presence of N-terminal signal peptide sequences in the identified proteins by

InterProScan and SignalP suites, and (4) prediction of potential virus-host interactions by analysing proteins expressed in hypertrophied salivary glands.

## *Profiling and identification of secretome proteins*

Electrophoretic profiles of saliva proteins harvested from asymptomatic and symptomatic flies are shown in **Figure 1**. It should be noted that equal amounts of proteins from the asymptomatic and symptomatic flies were loaded on into the SDS-PAGE gels. The proteins ranged from < 10 kDa to > 170 kDa in size. A gradual decrease (from 0 to 96 hpf) was observed in protein quantities for asymptomatic flies compared to a maximal quantity at 72 hpf for symptomatic flies. In addition, a (multiple) high intensity protein band(s) was observed in the 26 kDa region in the protein profile of asymptomatic flies relative to the symptomatic flies. At all the time points, majority of protein bands in approximately the 17-26 kDa, 29-43 kDa, 55-70 kDa and > 95 kDa range present in the secretome of symptomatic flies are absent in that of asymptomatic.

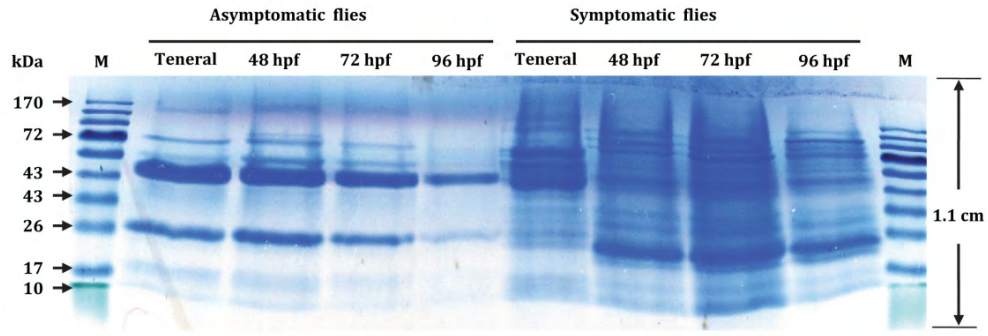


**Figure 1: *G. pallidipes* salivary gland secretome profiles:** CBB-stained SDS-PAGE gel showing the profiles of saliva proteins from asymptomatic (lanes 2-5) and symptomatic (lanes 6-9) *G. pallidipes*. The saliva was harvested at zero hpf (Teneral), 48 hpf, 72 hpf and 96 hpf. Lanes 1 and 10 were loaded with two µl of Pre-stained protein marker (Fermentas).

Analyses of the MS/MS data using the MaxQuant/Andromeda suites yielded 521 protein hits, 31 of which were virally encoded. As described in the materials and methods section above, the MS/MS data was filtered at or below FDR limit of < 1% (at the peptide level), detection of at least two unique peptides per protein, and merged to result in identification of 317 proteins. Twenty-five of the identified proteins were GpSGHV-specific. As an additional criterion for data quality, mass filtering was used to ensure that only those identifications resulting from within 10-ppm mass accuracy of the precursor ions were further analysed (see materials and methods). Taken together, the stringency applied in the analysis of the MS/MS data indicates that the datasets represented a high confidence catalogue of salivary proteins.

As can be observed in the protein profiles depicted in figure 1, the most significant differential protein expression profile was noted to be in the saliva harvested at 72 hpf. This time point was therefore selected as representative to investigate perturbations of protein expression pattern between asymptomatic and symptomatic flies (**Figure 2**). Of the proteins detected at 72 hpf, 39.4% (115/292) of host proteins and 52% (13/25) of the GpSGHV proteins were abundantly expressed in symptomatic flies relative to asymptomatic flies (**Figure 2 A**). While 14.4% (42/292) of host proteins showed relatively low abundance regardless of GpSGHV infection (**Figure 2 B**). In addition, 46.2% (135/292) and 48% (12/25) of host and GpSGHV proteins were specifically or uniquely expressed in the symptomatic salivary glands, respectively, compared to the asymptomatic flies (**Figure 2 C**).

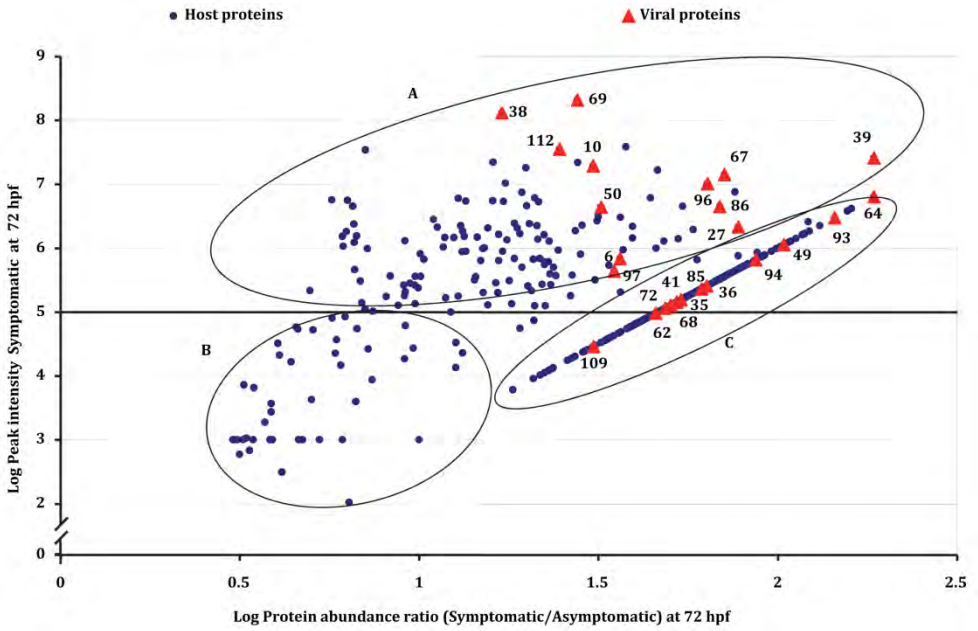


**Figure 2: Abundance ratios between saliva proteins harvested at 72 hpf from symptomatic and asymptomatic flies:** Host and viral proteins are indicated in blue dots and red triangles respectively. Groups A, B and C indicate the most abundantly expressed, least abundant, and proteins detected only in the symptomatic but not in asymptomatic flies respectively.

## *Structural and functional annotation of salivary gland secretome proteins*

InterProScan analysis of the identified proteins revealed that 96.5% (282/292) of host proteins and 24% (6/25) of viral proteins contained (predicted) secretion signals, respectively. Further, SignalP analysis of these proteins confirmed that 23% (65/282) of these host proteins and 24% (6/25) of GpSGHV proteins contained N-terminal

signal peptide sequences. The 65 saliva proteins (**Table 1**) are hereafter referred to as the salivary gland secretome proteins.

**Table 1: Sixty-five *Glossina pallidipes* salivary gland secretome proteins identified by LC-MS/MS**

| Contig ID** | Accession No. | Protein Name | Mol. Wt (kDa) | Unique peptides | Conserved domain |
|---|---|---|---|---|---|
| cn11113 | ADD18584.1 | SG growth factor 1 | 5.388 | 2 | |
| cn13111 | AAL87009.1 | Protease-Inhibitor | 8.6228 | 2 | SBPI/Kunitz |
| cn699 | ADD20212.1 | Niemann-Pick (TypeC) | 17.307 | 6 | MD-2-related lipid recognition |
| cn128 | XP_002056444.1 | Peptidase-S1/S6 | 17.739 | 3 | Tryp_SPc |
| cn7404 | CBA11306.1 | Pheromone/Odorant-binding-protein-99b-precursor | 17.9 | 6 | PBP/GOBP |
| cn13435 | CBA11325.1 | General odorant-binding-protein | 17.923 | 2 | PBP/GOBP |
| Gmm-2145 | ADD20435.1 | Hypothetical conserved protein | 18.229 | 2 | Metallophosphatase (MPP) & 5'-Nucleosidase |
| GMsg-6444 | ADD20479.1 | Nucleoside diphosphate kinase | 19.313 | 7 | NDPks |
| cn3611 | ADD20496.1 | Prostaglandin E2-synthase3/Hsp90 co-chaperone p23 | 20.951 | 5 | A-crystallin-Hsp_p23-like |
| cn9015 | ADD18444.1 | Profilin protein chickadee | 23.823 | 2 | PROF |
| GMsg-8947 | ADD19876.1 | Secreted phosphatidylethanolamine-binding protein | 24.441 | 4 | PEBP |
| cn4289 | ADD19264.1 | $Cu^{2+}Zn^{2+}$-superoide dismutase | 25.177 | 2 | SOD (P-class) |
| cn7661 | CAP78961.1 | Antimicrobial peptide Attacin B | 26.581 | 2 | Attacin_N & C-terminal |
| Gmm-3045 | ADD19954.1 | Hypothetical protein | 26.589 | 3 | |
| Gmm-2613 | ADD19951.1 | Hypothetical protein | 26.784 | 6 | |
| cn15528 | ADD19811.1 | $Ca^{2+}$-/Calmodulin-/Calcyphosine-like-protein | 26.992 | 5 | EF-hand, $Ca^{2+}$ binding motif |
| GMsg-7136 | ADD20155.1 | **Γ**-interferon inducible lysososmal thiol reductase | 27.011 | 2 | GILT |
| cn9673 | ADD18879.1 | Salivary Antigen 5 precursor | 28.901 | 2 | SCP-like extracellular protein |
| cn513 | ADD19989.1 | Salivary antigen 5-precursor variant | 28.909 | 17 | SCP_CRISP |
| Gmm-3046 | ADD19043.1 | Tsal2 protein precursor | 29.127 | 4 | NUC |
| cn2771 | ABC48941.1 | Lipophorin | 30.01 | 4 | Lipoprotein (LPD_N) |
| cn4297 | XP_002057612.1 | TEP2 protein precursor | 31.24 | 2 | α-2-macroglobulin (A2M_N) receptor |
| cn2281 | NP_523506.1 | TEP2 protein, IsoformA | 31.446 | 3 | ISOPREN_C2_like |
| cn9192 | ADD18265.1 | Hexamerin F1 (LSP-2) | 34.798 | 7 | Hemocyanin_N-& _M |
| cn399 | ADD18704.1 | Serine protease inhibitor | 36.032 | 2 | Tryp_SPc |
| cn6238 | ADD19820.1 | Trehalose-6-phosphate-synthas (component TPS1) | 37.979 | 2 | α-β-Haloacid Dehalogenase (HAD-SIF-IIB) |
| cn2477 | CAQ53422.1 | NTPase-/Torsin-like-protein | 38.032 | 2 | Walker A &B motifs |
| GLAFD01TV | XP_002048727.1 | Quiescin sulfhydryl oxidase4 | 38.377 | 6 | PDO |
| cn291 | ADD19085.1 | Major royal jelly protein | 38.685 | 3 | MRJP |
| cn1577 | ADD18617.1 | Trypsin | 39.789 | 2 | Tryp_SPc |
| cn8256 | ADD19420.1 | Fat body Chymotrypsin | 39.868 | 2 | Tryp_SPc |
| GMsg-7644 | ADD19393.1 | Secreted angiopoietin-like protein | 39.95 | 2 | FReD |
| cn3048 | ADD18624.1 | DnaJ/Hsp40 protein | 40.013 | 5 | DnaJ-C |
| cn8409 | ADD18624.1 | Molecular chaperon | 42.936 | 7 | DnaJ |
| cn3041 | ADD18511.1 | Vacuolar ATPase sector accessory subunit-S1 Ac45 | 43.367 | 3 | ATP-synt_S1 |
| cn1049 | ABN58709.1 | Tsal2-protein-Isoform A | 43.992 | 11 | NUC |
| cn43 | Q2PQM7.1 | Chitinase-like-protein | 44.986 | 7 | GH18_Chitinase-like |
| cn4273 | ADD18566.1 | Large serine protease | 46.844 | 3 | Tryp_SPc |
| cn408 | ADD18797.1 | Calreticulin precursor | 46.94 | 5 | Calreticulin |

| Contig ID** | Accession No. | Protein Name | Mol. Wt (kDa) | Unique peptides | Conserved domain |
|---|---|---|---|---|---|
| cn8210 | ABC25072.1 | Serine protease inhibitor-1 | 46.971 | 3 | SERPIN |
| cn6032 | ADD19384.1 | Medium-chain-Specific-acyl-CoA dehydrogenase (mitochondrial) | 47.307 | 4 | ACAD/CaiA |
| cn8313 | ADD19747.1 | Yellow precursor | 47.51 | 5 | MJRP |
| cn2784 | ABC25079.1 | Serine protease inhibitor | 48.605 | 9 | SERPIN |
| cn3205 | ADD18309.1 | Chitinase–like protein-precursor | 51.254 | 17 | Glyco_hydro_18 |
| cn1718 | ADD18950.1 | Inorganic pyrophosphatase/nucleoside remodelling factor subunit NURF3 | 51.403 | 3 | Pyrophosphatase |
| cn2284 | ADD19233.1 | Yellow-f | 52.034 | 5 | MRJP |
| cn4622 | ABC25095.1 | Imaginal growth factor-3 | 52.469 | 2 | IDGF |
| cn8091 | ADD18562.1 | Translation Initiation factor 5C (eIF4-γ/eIF5/eIF2-ε | 52.936 | 2 | W2 |
| cn4758 | ABC25074.1 | Serine protease inhibitor-4 | 53.656 | 3 | SERPIN |
| cn16500 | XP_002046796.1 | Hypothetical conserved protein | 53.731 | 3 | Glyco_hydrolase-16 |
| GMsg-8203 | ABN80093.1 | Ecto-5'-nucleotidase-related protein | 53.977 | 5 | MPP_CD-73_N |
| cn13608 | Q2PQN0.1 | Chitinase-like protein Idgf1 | 54.435 | 2 | GH18_(IGDF1) _chitnase-like |
| Gmm-3154 | ADD18584.1 | Adenosine/AMP deaminase-related-growth factor C | 57.003 | 32 | Adm_rel/Metallo-dependent hydrolase |
| cn7518 | ADD20206.1 | Lectin | 57.87 | 9 | CLECT |
| cn786 | ADD20271.1 | Protein disulfide isomerase | 59.046 | 5 | ER_PDI-(a,a',b & b' subfamilies) |
| cn3338 | ADD20489.1 | Aldehyde dehydrogenase | 59.145 | 7 | ALDH_F7/SF_AASADH/δ-1pyr5carbox2 |
| cn12530 | ADD20435.1 | 5'-nucleotidase-related-salivary protein | 61.603 | 7 | MPP_CD73_N |
| cn583 | ADD20425.1 | Carboxyesterase | 63.125 | 8 | COesterase_lipase |
| cn2587 | ADD18753.1 | Homogentisate 1,2-dixoygenase | 63.875 | 2 | HgmA |
| cn14986 | XP_002002462.1 | Angiotensin- converting enzyme | 69.118 | 7 | GluZincin/Peptidase_M2 |
| cn1859 | ADD19839.1 | Hypothetical conserved protein | 69.181 | 11 | XendoU |
| GMsg-8257 | ADD20246.1 | Heo1-protein | 71.965 | 6 | GH20_hexosaminidase |
| cn15340 | ADD20300.1 | Hsp70 cognate 3 | 72.657 | 5 | Hydantoinase/oxoprolinase |
| cn747 | XP_002002903.1 | Peptidase family M2 Angiotensin converting enzyme-related-protein-precursor | 73.179 | 2 | M2_AC |
| cn270 | AAM46784.3 | Transferrin | 75.362 | 12 | Transferrin |

** Contig ID obtained from the *G. m. morsitans* salivary glands expressed sequence tags (ESTs) library available at: http://old.genedb.org/genedb/glossina/.

Twenty of the 65 salivary gland secretome proteins were only detectable in the symptomatic as opposed to the asymptomatic flies, i.e. the twenty proteins were not detectable in the asymptomatic flies (**Table 2**).

**Table 2: Twenty host proteins detected in saliva harvested from symptomatic flies but were not detectable in the saliva that was harvested from asymptomatic flies.**

| Mol. mass range | Protein Name | Functional annotation |
|---|---|---|
| < 26 kDa | Niemann-Pick TypeC-2 | Involved in mesoderm development |
| | Attacin AttA | Involved in humoral immune response |
| | Protease-inhibitor-SBPI | Serine-type endopeptidase inhibitor |
| | Calcyphosine-like-protein | $Ca^{2+}$-binding protein involved in phagocytosis |
| | ProstaglandinE-synthase 3 | |
| | Hypothetical conserved protein | Contains coiled-coil region |
| | Putative-LD47508p | Gamma-interferon-inducible lysosomal thiol reductase |

| Mol. mass range | Protein Name | Functional annotation |
|---|---|---|
| | Cu/Zn-superoide dismutase | Superoxide dismutase involved in Redox process |
| 26–43 kDa | LD13269p | Serine-type endopeptidase (proteolysis) |
| | TEP2-protein precursor | Endopeptidase inhibitor (humoral immune response) |
| | Torsin-like-protein-precursor | chaperone-mediated protein folding in |
| | Major-royal-jelly-protein | Yellow protein precursor |
| | Vacuolar-ATP-synthase-subunit S1 | $H^+$-transporting V-type ATPase involved in rotational mechanisms |
| | S-adenosylmethionine synthetase | Cytoplasmic ATP-binding protein involved in small GTPase-mediated signal transduction and S-adenosylmethionine biosynthesis |
| | Trehalose-6-phosphate-synthase | Catalysis in trehalose biosynthetic process |
| | D-isomer-Specific-2-hydroxyacid-dehydrogenase | NADH binding oxidoreductase |
| | Quiescin-sulfhydryl oxidase4 | A thiol-oxidase involved in cell redox homeostasis |
| | Angiopoietin-like protein | Involved in receptor binding during signal transduction |
| 55-70 kDa | Juvenile hormone esterase | Hydrolase |
| | Angiotensin-converting-enzyme | Membrane-located metallopeptidase |
| | Hexosaminidase isoform A | A plasma membrane β-N-acetylglucosaminidase |

The twenty-five GpSGHV-encoded proteins were detected in the saliva harvested from the symptomatic flies (**Table 3**).

**Table 3: Twenty-five GpSGHV-encoded proteins detected in the *G. pallidipes* hypertrophied salivary glands**

| ORF # | Mol. Wt (kDa) | Putative Protein Name | Signature domain | Functional annotation or characteristics | References |
|---|---|---|---|---|---|
| 6 | 41.678 | Lecithin:cholesterol acyltransferase (LACT) | α/β hydrolase catalytic domain | Membrane-docking (to NPC); capsid processing (nucleocytoplasmic export) | (Chizmadzhev, 2004; Greber and Puntener, 2009; Jovasevic *et al.*, 2008; Mabit *et al.*, 2002; Smede *et al.*, 2009) |
| 10 | 127.03 | Spectrin | Spectrin repeats | Involved in cytoskeletal structure; anterograde protein trafficking | (De Matteis and Morrow, 1998; De Matteis and Morrow, 2000) |
| 27 | 53.064 | Chitinase-II | Chitinases family 18 active site (*O*-Glycosyl hydrolases) | Virus attachment to host cells and cell lysis | (Bisaillon *et al.*, 1999; Hiramatsu *et al.*, 1999a; Saville *et al.*, 2004) |
| 35 | 10.424 | Thymidylate synthase | Thymidylate synthase Active site | Regulation of balanced supply of dNTPs during DNA replication | (Gribaudo *et al.*, 2000; Gribaudo *et al.*, 2002) |
| 36 | 13.794 | deoxycytidylate hydroxymethylase (dCMP-HMase) | Thymidylate synthase, chain A | Viral DNA replication (pyrimidine metabolism; expressed "early" during viral infection) | (Cohen, 1972; Lewis and Cohen, 1963) |
| 38 | 136.55 | Maltodextrin glycosyltransferase | α Amylsase catalytic domain (α-β barrel containing active site) | Possible involvement in the expression of receptor protein for transport across cell membrane | (Meyer *et al.*, 2010) |
| 39 | 37.659 | HSP90-like APTase (HATPase_C ) | TM, SP; Gyrase, Hsp90, Histidine-Kinase, Mutl (GHKL) domain | Signaling; regulation of DNA supercoiling/(un) winding of DNA strands | (Vozzolo *et al.*, 2010) |
| 41 | 48.773 | Casein kinase isoform I-δ | Protein kinase domain | Establishment of directional movement of encapsidated viral genome by phosphorylating cytoskeletal components | (Granzow *et al.*, 2004; Smith and Enquist, 2002) |
| 46 | 61.536 | | | | |
| 49 | 10.009 | Glutathione S-transferase<br>Pre-mRNA Splicing factor 9-like protein | WD40 repeat, G-β-repeat | Possible signalling (to circumvent host defence mechanism)<br>PRP19-associated complex; Associates with spliceosome; Control production of ion-channel protein | (Shih and Krug, 1996) |
| 50 | 32.741 | Uncharacterized | α Helix-β stand-α helix | | |

| ORF # | Mol. Wt (kDa) | Putative Protein Name | Signature domain | Functional annotation or characteristics | References |
|---|---|---|---|---|---|
| 62 | 512.12 | p53 transcription factor-like | β-sandwich domain of Sec23/24 superfamily | Chromosome segregation (induced "early" in S-phase); p53 suppressor protein | (Dahl *et al.*, 2005; Dobner *et al.*, 1996; Moore *et al.*, 1996; Turpin *et al.*, 2005) |
| 64 | 70.023 | ATP-binding cassette-type-(ABC ATPase) like protein | Zinc Finger domain | Translation initiation, ribosome biogenesis and capsid assembly | (Hakim and Fass, 2009; Pisarev *et al.*, 2010; Rodnina, 2010) |
| 67 | 31.019 | Uncharacterized | α Helix-β stand-α helix | | |
| 68 | 12.651 | Riboflavin uptake protein, chain a (ECF Transporter) | | Membrane-embedded (cellular component); binds to Riboflavin | (Shelby and Webb, 1997) |
| 69 | 30.914 | $Ca^{2+}$ and $Zn^{2+}$ – binding protein | TM, SP | | |
| 72 | 31.771 | FAD-dependent sulfhydryl oxidase | TM | Involved in virion maturation (virion assembly; expressed "late" in infection cycle) | (Hakim and Fass, 2009) |
| 85 | 30.09 | Signaling Protein | α Helix-β stand-α helix | Tyrosine kinase-dependent signalling; Transport of viral structural proteins | (Lin *et al.*, 1984) |
| 86 | 70.176 | Uncharacterized | α Helix-β stand-α helix | | |
| 93 | 38.532 | Hydrolase (taype-IIe restriction enzyme) | TM | DNA-binding & cleavage | (Xia *et al.*, 1986) |
| 94 | 32.70 | | α Helix-β stand-α helix | | |
| 96 | 43.527 | Metal-binding protein (transport channel protein) | Multiple- TM, SP | $Mn^{2+}$ & $S_2O_4^{2-}$ -binding | (Takeuchi and Lamb, 1994) |
| 97 | 44.403 | Vesicle-associated membrane (exocytosis) protein | TM | Involved in targeting and/or fusion of vesicles to target membrane | (Mollinedo *et al.*, 2003) |
| 109 | 33.194 | Putative uncharacterized protein | Cystathionine-β-synthase (CBS) domain) | | (Chavali and Ghosh, 2007) |
| 112 | 19.057 | Regulatory protein | Helix-turn-helix | Association with PML nuclear bodies | (Everett, 2001) |

TM = (Hydrophobic) trans-membrane domain; SP = Signal peptide.

At least one gene ontology (GO) term could be assigned to 285 of the 317 detected saliva proteins. Only five (1.8%) of the host proteins were deemed of unknown function after blast searches against the nr databases and annotation augmentation. Based on the GO annotations, the MS/MS-supported proteins could be grouped into three categories: (1) the broad biological processes the proteins are involved in, (2) the predicted molecular functions they perform, and (3) the sub-cellular structures complexes or components these proteins associate with (**Figure 3**). The three most common biological processes were metabolic (24.7%), cellular (23%) and biological regulation (10.1%), whereas the three most common molecular functions were nucleotide/nucleoside-binding (combined percentage of 30.7%), hydrolase activity (21.7%), protein binding (16.2%) and transferase activity (13.2%). The multilevel cellular components analysis returned five sub-categories, of which the highest proportion (27.2%) of the proteins showed association with lipid-related metabolism (**Figure 3**).

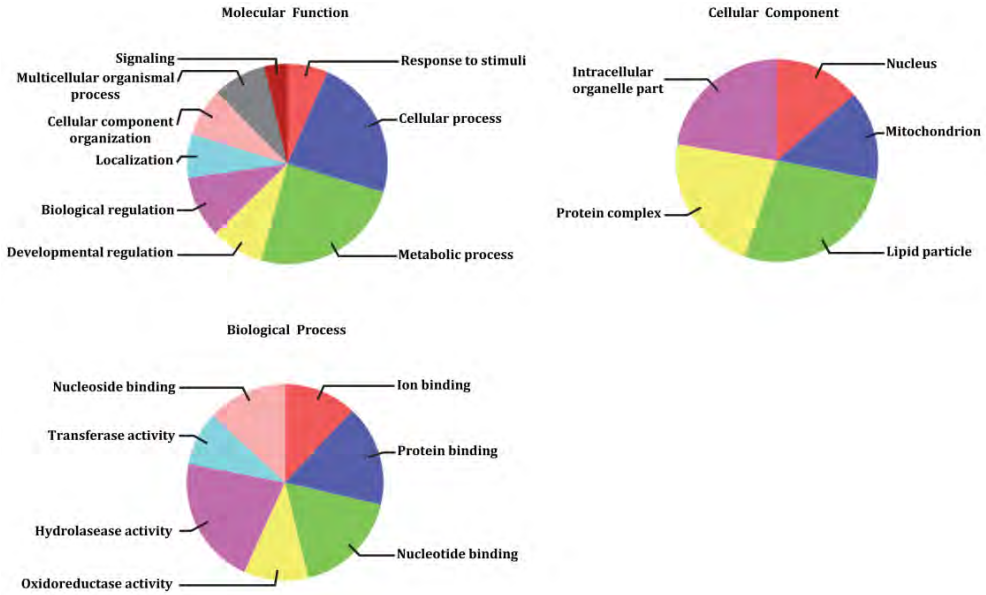


**Figure 3: Classification of *G. pallidipes* secretome proteins:** Gene Ontology (GO) terms for categorization of salivary gland secretome proteins by molecular function, biological process and cellular component.

# Discussion

## *Importance of tsetse fly's salivary glands in the transmission and dissemination of GpSGHV*

The general feeding behaviour of an insect vector may aid in the transmission and dissemination of an insect virus from an infected to a non-infected host. For instance, in some plant feeding insects such as the aphids, the components of the insect's saliva may increase virus transmission in and between plants hosts during the insect's sampling probes without the virus being circulated in the peripheral vascular system of the insect vector (Ng and Falk, 2006). In the case of hytrosaviruses (GpSGHV and MdSGHV), the replication of the virus in the insect's salivary glands, which is the primary replication organ for the viruses (Garcia-Maruniak *et al*., 2009), could be advantageous for the virus because accumulation of the virus in the salivary glands could promote the horizontal virus transmission of the virus from an infected to uninfected tsetse fly host via saliva secretions. Replication and accumulation of the viruses in the salivary glands probably ensure the transmission and dissemination of these viruses to as many susceptible hosts as possible.

Like many arthropods, tsetse fly saliva contains a complex mixture of proteins and peptides. Therefore, for efficient transmission, GpSGHV must have ability to survive or exploit biological (immune-modulatory) activities of the proteins and macromolecules present in the host's in the saliva. Potentially, specific salivary gland proteins in GpSGHV-infected tsetse flies may increase the chances of blood-feeding and virus transmission by infected tsetse flies. In the following sections, the major *G. pallidipes* secretome proteins are broadly discussed in terms of their functions. It should be noted that the discussions are based on studies that have been performed in other virus-insect systems. Further, the term 'protein abundances' refers to the overall number of peptide ion abundances identified as coming from a specific protein. This is important because, despite SDS-PAGE gel electrophoresis step (which could be considered as a protein purification step before LC-MS/MS analysis), the protein samples used in this study were rather crude. Purification of the proteins samples would probably result to fewer but higher peptide concentrations present in each sample. The analysis presented in this chapter depicts relative protein quantification only. The abundances of the identified peptides/proteins were normalized against their relative molecular weights.

## *Identification and characterization of G. pallidipes secretome proteins*

The proteins in the saliva of *G. pallidipes* were identified and characterized using LC-MS/MS on tryptic peptides obtained from SDS-PAGE separated salivary proteins (in-gel samples). In total, 521 well-supported proteins were obtained in searches against the *Glossina* EST and genomic databases. Possibly, the number of the proteins would have been greater if the stringent post processing filters used in the MaxQuant/Andromeda had been relaxed, and if all peptides with a single unique peptide hits had been considered.

Sixty-five of the identified salivary gland proteins had N-terminal peptide sequences, implying that they are bona fide secretome proteins. Although these data are insufficient to provide insights into the roles of the identified proteins, potential effector roles of many of the proteins in host-virus interactions can be predicted based on their homologies to other proteins with known functions. In general, the secretome proteins identified in this study could be grouped into six (predicted) functional categories, some of which are discussed in the following sections.

## *Protein folding machinery*

Heat shock proteins (HSPs) were detected in high abundance in the salivary gland secretome of symptomatic flies relative to the asymptomatic flies. Many viruses are known to interact with HSPs at different infection stages. For instance, in Epstein - Barr virus (EBV), viral attachment to cell membrane receptors activates signal

transduction pathways involving heat shock responses (Cheung and Dosch, 1993). HSPs are also involved in viral cell entry (Lopez and Arias, 2004; Perez-Vargas *et al.*, 2006; Zarate *et al.*, 2003), and internalization of viruses into host cells (Chroboczek *et al.*, 2003; Chromy *et al.*, 2006; Greene and Eisenberg, 1990; Niewiarowska *et al.*, 1992; Rapoport *et al.*, 2008; Saphire *et al.*, 2000; Ungewickell, 1985). Viral proteins, including *E1A* of Adenovirus (Phillips *et al.*, 1991), large T antigen (*T* ag) of Simian vacuolating virus 40 (Kingston *et al.*, 1986; Simon *et al.*, 1988), ICP4 of Human simplex virus, *IE2* of human cytomegalovirus, and nuclear antigen 3 (*EBNA3*) of EBV (Caswell *et al.*, 1993; 1996; Colberg-Poley *et al.*, 1992; Furnari *et al.*, 1993; Hagemeier *et al.*, 1994; Young *et al.*, 2008), modulate HSPs by direct interaction with different components of the basal transcription apparatus. Additionally HSPs play important roles in suppression of O'nyong-nyong virus replication and in the establishment of latent infections in the mosquito *Anopheles gambiae* (Sim *et al.*, 2005; 2007).

### *Pathogen recognition and defence response proteins*

C-type lectins (CTLs) were detected in higher abundance in the salivary secretome of the symptomatic compared to asymptomatic flies. CTLs are known to recognize pathogen-associated molecular patterns (PAMPs) (Watanabe *et al.*, 2006; Weis *et al.*, 1998) in invading pathogens (Janeway, Jr., 1989; Medzhitov and Janeway, Jr., 1997). The members of thioester-containing proteins (TEPs), which have been described in the complement system of *Drosophila melanogaster* and *An. gambiae* (Blandin et al., 2004), were also detected only in the secretome of symptomatic flies (**Table 2**). It is likely that the symptomatic flies express CTLs in the salivary glands to block glycoprotein-mediated attachment of GpSGHV to non-infected cells. Although CTLs may diffuse viral infection, their binding to circulating virus could effectively reduce viral infection of lectin-expressing cells.

### *Protein export machinery*

ADP-ribosylation factor (ARF) is an abundant protein that reversibly associates with Golgi membranes, and is implicated in the regulation of membrane traffic through the secretory pathway (D'Orsogna and Chou, 2009; Hall, 1990; Stearns *et al.*, 1990). This pathway is important for processing of viral contents into complexes capable of nuclear penetration. ARFs have been shown to be up-regulated and involved in virus infection (Belov *et al.*, 2007; Ma *et al.*, 2010; Zhang *et al.*, 2010), and possibly explains the detection of ARF in the saliva of symptomatic flies harvested at 48, 72 and 96 hpf, as opposed to asymptomatic and teneral-symptomatic flies at 0 hpf. This result suggests recruitment of ARFs to membranes by GpSGHV and may provide clues for future studies on replication pathway.

## *Proteases and protease inhibitors*

Six types of serine proteases were up regulated in the secretome of symptomatic flies compared to the asymptomatic (**Tables 1** and **2**). Also detected was a precursor of phosphenol oxidase activating factor of the prophenol-oxidase-activating system (proPO-AS), an important component of insect innate immune defence (Cerenius and Soderhall, 2004; Franssens *et al.*, 2008; Kanost *et al.*, 2004). These two proteins, together with the TEPs described above, are involved in initiating a signal cascade that culminates in melanisation, which includes the formation of toxic intermediary compounds to kill invading pathogens (Franssens *et al.*, 2008). In addition, the up-regulation of the serine proteases in the symptomatic flies may implicate their involvement in the activation of viral proteins during the infection process. Studies have shown that the baculovirus P74, a viral attachment protein (Faulkner *et al.*, 1997; Haas-Stapleton *et al.*, 2004; Yao *et al.*, 2004), is cleaved by trypsin to enable interaction with host receptors. The GpSGHV P74, though detected in very low abundance, is probably required for the interaction with the host receptors, and may be activated by host-derived proteases.

## *Housekeeping genes*

RNA polymerase II general transcription factor (BTF3), translation elongation factor EF-1 gamma (EF1γ), and ATP-dependent RNA helicase were up regulated in the secretome of symptomatic flies compared to the asymptomatic. BTF3 is a general transcription factor necessary for activation of a number of viral promoters by RNAP II (Moncollin *et al.*, 1992), while EF1γ is involved in the regulation of protein assembly and folding (Caldas *et al.*, 2000; Koonin *et al.*, 1994). The detection of these proteins in symptomatic flies, coupled to the presence of RNA helicase is desirable for the expression of replication- and maturation-related genes. Proteins involved in signal transduction were also detected (in saliva harvested at 48, 72 and 96 hpf), including GTPase-activating protein (GAP), cAMP-dependent protein kinase, and Ras-related small GTPase (Rho type). GAP is known to be necessary for efficient virus infection and replication (Sklan *et al.*, 2007), and is implicated in the regulation of anterograde traffic between the ER and the Golgi complex, while cAMP-dependent protein kinase is implicated in the regulation of virus infection and virus-induced cell-cell fusion.

## *Cytoskeletal proteins*

Most viruses use components of the host cytoskeleton to move within cells. Upon virus infection, virions or sub-viral nucleoprotein complexes are transported from the cell surface to the site of viral transcription and replication. During egress, viral particles containing proteins and nucleic acids move again from the site of their synthesis to that of viral assembly and further to the plasma membrane (Campbell

and Hope, 2005; Dohner and Sodeik, 2005). Viral (sub) particles, particularly in members of herpesviridae, adenoviridae, parvoviridae, poxviridae and baculoviridae are known to use the microtubule and/or the actin cytoskeleton. In this study, actin 5C, actin 87E and actin depolymerizing factor were detected in all saliva samples except in the asymptomatic flies 96 hpf. F-actin capping protein was detected in the saliva of symptomatic flies at 48 hpf, while actin 57B was detected, albeit in low abundance, in symptomatic flies at 72 hpf. Myosin heavy chain, which drives transport along actin filaments (Campbell and Hope, 2005), was detected in the saliva of symptomatic flies except at 96 hpf, which probably indicates reduced active transport of virions at this time point. While all the six cytoskeletal proteins were detected in the saliva at 96 hpf, none was detected in the secretome of asymptomatic flies 96 hpf.

## *(Predicted) annotation of GpSGHV proteins detected in G. pallidipes salivary secretome*

Viral replication in host insect requires specific interactions between viruses and host components. DNA viruses have evolved mechanisms to evade the host restrictions at entry, cytoplasmic transport, replication, protein synthesis, innate immune recognition, and egress. In this study, structural and functional annotations of the identified GpSGHV proteins indicate their engagement with host cellular metabolism.

### Viral entry into host cells

The stepwise entry of DNA viruses into host cells requires viral attachment to cell surface receptors and lateral movements of the virus-receptor complex to specialized sites on the plasma membrane (Dugan *et al.*, 2006; Greber *et al.*, 1993; 2002; 2007). In the baculoviruses, PIF proteins have been shown to be involved in viral attachment (Slack *et al.*, 2001). In the current study, homologs of the baculovirus PIFs were detected. In addition, the GpSGHV ORF85 product detected in this study, is a tyrosine kinase-dependent signalling protein, and is probably involved in transport of viral polypeptides into the nucleus. Such transport of viral proteins has been documented in SV40 (Lin *et al.*, 1984). Additionally, viruses in general elicit signals following attachment to the host cell membrane to circumvent the host defence mechanism. In this regard, the GpSGHV ORF046 product is a glutathione S-transferase-like protein, thus pointing to its possible involvement in this type of signalling.

### Bidirectional cytoplasmic transport of viral particles

Nuclear replicating viruses use microtubule motors for trafficking towards the nucleus and the periphery during egress (Greber, 2005; Lin *et al.*, 1984; Marek *et al.*, 2011). Bidirectional transport allows precise delivery of capsids to ensure nuclear targeting, and has been demonstrated in HSV-1 (Mabit *et al.*, 2002; Ohkawa *et al.*,

2010) and in human adenovirus 2/5 (Ad2/5) (Kelkar *et al.*, 2004; Suomalainen *et al.*, 1999; 2001). Incoming DNA viruses expose proteins on the capsid that preferentially recruit microtubule motor complexes (Greber, 2005), and may release tegument proteins before they traffic to the nucleus (Luxton *et al.*, 2005). To regulate capsid transport, protein phosphorylation by viral and/or host cellular kinases modulate tegument protein composition (Newsome *et al.*, 2004). In this study, the detected cAMP-dependent protein kinase is probably involved in anterograde trafficking of GpSGHV. Additionally, the GpSGHV ORF041 product is a casein kinase, which is likely to be involved in phosphorylating cytoskeletal components both in anterograde and in egress (Granzow *et al.*, 2004; Smith and Enquist, 2002). Early during infection, some viruses such as δ-2 human herpes virus-8 and hepatitis C virus induce Rho GTPases (Naranatt *et al.*, 2005; Sklan *et al.*, 2007), which alter the dynamics by increasing the acetylation of actin microfilaments thereby enhancing viral capsid trafficking transport to the nucleus and establishment of successful infection. Ras-related small GTPase (Rho type) as well as GTPase-activating protein (GAP) were detected in saliva harvested from symptomatic flies, and suggest their participation in viral trafficking towards the nucleus. Finally, although the role of spectrins in cytoplasmic transport is not clear, this study identified the product of GpSGHV ORF010 to have spectrin repeat domains, indicating its potential involvement in GpSGHV anterograde trafficking.

### Docking, uncoating and release of viral DNA into nucleus

Cytoplasmic transport is followed by viral genome docking and uncoating at the nuclear pore complex (NPC), a stepwise programme involving partial proteome degradation of incoming capsid or tegument proteins (Delboy *et al.*, 2008; Wolfstein *et al.*, 2006). Although it is not clear how uncoating at the NPC occurs, experiments with some viruses such as herpes B virus have indicated that, capsids are transported to the nuclear membrane where they bind to NPCs and release their genome into the nucleus (Rabe *et al.*, 2006). Additionally, cytoplasmic processing of incoming capsids makes them competent for docking to the NPC (Meyer *et al.*, 2010), and probably prevents the naked viral chromatin from traveling through the cytoplasm, which could trigger DNA-sensing host innate immune responses as has been demonstrated in adenovirus (Muruve *et al.*, 2008). The protein encoded by GpSGHV ORF006 has a α/β-hydrolase catalytic domain, a signature domain for lecithin-cholesterol acyltransferase (LACT), which is involved in membrane docking of viruses to NPC, as well as in nucleocytoplasmic transport of capsids.

### Development of viral transcription and replication

Upon infection, some viruses such as the baculovirus *Autographa californica* multi-nucleopolyhedrovirus establish centres for transcription, DNA replication and progeny nucleocapsid assembly, and others express at least one regulatory protein

that interacts directly with similar domains such as the promyelocytic leukemia protein nuclear bodies (PML-NBs) (Everett, 2001). Annotation of the GpSGHV ORF112 product revealed presence of the helix-turn-helix characteristic domains of regulatory proteins that may be involved in DNA-protein interactions. In addition, gamma-interferon was detected in the saliva harvested from symptomatic flies at 48 hpf, as well as a 19.3 kDa host protein encoded by GMsg-6444. The GMsg-6444 is an ubiquitin-like protein, a partner protein to viral replication centre and is dramatically enhanced by interferon (Lallemand-Breitenbach and de Thé, 2010). Viral proteins associating with these centres have the ability to stimulate lytic infection and induction of reaction from quiescence (Everett *et al.*, 1998). The detection of these proteins is significance: under yet undefined environmental and/or genetic conditions, the asymptomaticGpSGHV infection state (represented by majority of the flies in tsetse fly colonies, can convert to the symptomatic state (see **Chapter 6**). Also detected in this study were products of GpSGHV ORFs 35 and 36, homologs of thymidylate synthase and deoxycytidylate hedroxymethylase, respectively. The former is involved in regulating a balanced supply of dNTPs during DNA replication (Gribaudo *et al.*, 2000; 2002), while the latter is involved in pyrimidine metabolism (Cohen, 1972; Lewis and Cohen, 1963). Further, the product of GpSGHV ORF039 (a HSP90-like ATPase) detected in this study, is possibly involved in regulation of unwinding of DNA supercoil strands. Additionally, the GpSGHV ORF062 product, a p53 transcription factor-like protein containing β-sandwich domain of the sec23/24 super-family, was detected. Proteins of this family are involved in chromosomal segregation (Dahl *et al.*, 2005; Dobner *et al.*, 1996; Moore *et al.*, 1996; Turpin *et al.*, 2005), and have direct roles in viral DNA transcription and replication. Also detected in this study was an ABC ATPase-like family protein (product of GpSGHV ORF064). Studies have implicated members of this protein family to be involved in translation initiation, ribosome biosynthesis and virus capsid assembly of other viruses such as HIV (Pisarev *et al.*, 2010; Rodnina, 2010). Taken together, the presence of these host and GpSGHV proteins in the symptomatic flies appear to suggest that GpSGHV genome associates with the periphery of PML-NBs, and that viral replication compartments would develop from these sites.

### Viral maturation and nuclear egress

The product of GpSGHV ORF049 detected in this study was predicted to be a pre-mRNA splicing factor-9-like protein. The WD40/G-β-repeats present in this protein are a signature domain for proteins that associate with the spliceosome (Shih and Krug, 1996). Other detected proteins that have potential roles in GpSGHV maturation were: (i) products of GpSGHV ORF072, a FAD-dependent sulfhydryl oxidase (with a late promoter motif and hence likely to be involved in virion maturation; (Hakim and Fass, 2009)); (ii) ORF093, an uncharacterized endonuclease type-IIe-like protein with DNA-binding and cleavage activities (Xia *et al.*, 1986); (iii) ORF027, a chitinase-II (*O*-

glycosyl hydrolase) protein, which like the chitinases family-18 proteins may be involved in virus maturation (See reviews by Bisaillon *et al.*, 1999; Hiramatsu *et al.*, 1999a; Saville *et al.*, 2004); (iv) ORF038, a protein containing α-β-barrel active site, thus likely to be involved in the expression of receptor proteins for membrane transport (egress) (Meyer *et al.*, 2010) and (v) ORF096, a metal ($Mn^{2+}$) and ion ($S_2O_4^{2-}$)-binding protein with multiple TMs, thus likely to be part of the viral ion-channel proteins. Newly assembled enveloped viruses recruit periphery directed motors, are transported to the plasma membrane on the microtubules upon binding of the outer membrane (Marek *et al.*, 2011) proteins, and fuse with plasma membrane. Although it is unclear whether GpSGHV travel in vesicles or as capsid as baculoviruses do, the product of GpSGHV ORF097 was predicted to be a vesicle-associated membrane protein and could be involved in targeting and/or fusion of virus-containing vesicles to the target membranes (Luxton *et al.*, 2005; Nagel *et al.*, 2008).

# Conclusions

Based on the data presented in this chapter and chapter 4, GpSGHV appears to trigger the modulation of both cellular and secreted proteins in *G. pallidipes* salivary glands. Overall, most regulated proteins may be over-expressed upon GpSGHV infection. It is tempting to assume that among the modulated salivary gland proteins, most may be in favour of the virus and its transmission, either through direct interactions, or by acting on the success of the blood-feeding process. The *G. pallidipes* salivary gland secretome encompasses a wide spectrum of proteins that may be required for the different facets of the GpSGHV infection cycle from viral attachment to egress of the virions from infected host cells. The GpSGHV alters the protein expression patterns in the host salivary glands. Whereas either some of the host proteins are up-or down-regulated, other proteins appear to be specifically expressed in the symptomatic flies compared to the asymptomatic.

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# Chapter 6

# Role of microbiome in GpSGHV trans-generational transmission[5]


## Abstract

Tsetse flies (Diptera: Glossinidae) harbour three known vertically transmitted bacterial symbionts (*Wigglesworthia*, *Sodalis*, and/or *Wolbachia*) that modulate the reproduction and immune competence of the flies. *Wigglesworthia* and *Sodalis* are both intra- and extra-cellular, while *Wolbachia* is strictly intracellular. *Wigglesworthia* resides in the midgut bacteriocytes or in the milk glands and does not infect reproductive tissues. *Sodalis* resides in the reproductive tract and infects the mid-guts, hemolymph and milk glands, while *Wolbachia* exclusively infects germ tissues. Some tsetse species such as *G. pallidipes* also harbour *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV), whose infection phenotypes can be either asymptomatic or symptomatic. In mosquitoes, aphids and tsetse the removal of the symbionts by antibiotics impacts host fitness and increases host susceptibility to pathogen infections. We therefore hypothesized that suppression of the *G. pallidipes* symbionts would alter GpSGHV titres and/or expression of salivary gland hypertrophy (SGH) in parent and their progenies. Injected virus replicated in the parents, but was not secreted via saliva during fly feeding. Whereas GpSGHV-injected parents did not exhibit SGH, the incidence of SGH symptoms increased from 4.5% to > 95% from the first to the fourth larviposition cycles of the $F_1$ progenies. Ampicillin had negligible impacts on the virus titres in adult parents. However, the antibiotic reduced titres of GpSGHV, *Sodalis* and *Wigglesworthia*, and negated expression of SGH symptoms in the $F_1$ progenies. *Wolbachia* was not detected in any of the analysed samples. The results indicated that the fat body tracheal system may provide a conduit for the trans-generational GpSGHV transmission via milk gland secretions, and that the removal of the microbiome suppresses this transmission. These data suggest that the *G. pallidipes* microbiome may have co-evolved with GpSGHV, and plays key roles in the mother-to-progeny transmission of the virus.


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# Introduction

Tsetse flies (Glossina; Diptera) harbour a highly regulated microbiome made up of three bacterial symbionts (obligate *Wigglesworthia glossinidia*, commensal *Sodalis glossinidius* and/or parasitic *Wolbachia pipientis*) that affects host's nutritional and reproductive fitness (Aksoy, 1995; Dale and Maudlin, 1999; Doudoumis *et al.*, 2012; O'Neill *et al.*, 1993; Wang *et al.*, 2013), immune competence and longevity (Saridaki and Bourtzis, 2010; Schneider *et al.*, 2011). Removal of the symbionts by antibiotic treatment can negatively influence the host's fitness (Alam *et al.*, 2011; Pais *et al.*, 2008). In tsetse flies, *Wigglesworthia* occurs in the bacteriocyte adjacent to the midgut or intra-cellular in the milk glands (Attardo *et al.*, 2008). *Sodalis* is found intra- and extra-cellular in the gut, hemolymph and salivary glands (Cheng and Aksoy, 1999), while *Wolbachia* localizes within germ-line tissues (Cheng *et al.*, 2000). Whereas *Wigglesworthia* and *Sodalis* are transmitted to the intrauterine progeny via milk gland secretions, *Wolbachia* is maternally transmitted via the egg cytoplasm (Stouthamer *et al.*, 1999). In the absence of *Wigglesworthia*, adult progenies are immune compromised (Pais *et al.*, 2008). Functional roles of *Sodalis* are unclear. *Wolbachia* is capable of rapid spread into insect populations using unidirectional cytoplasmic incompatibility (CI) (Bourtzis *et al.*, 2003), the imposition of crossing sterility that give females a reproductive advantage.

Tsetse flies are also infected by the *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV; family *Hytrosaviridae*) (Abd-Alla *et al.*, 2009b). GpSGHV is horizontally (Abd-Alla *et al.*, 2011b), and vertically transmitted either trans-ovum (Jura *et al.*, 1989) or via infected milk gland secretions (Sang *et al.*, 1996). In *G. pallidipes*, GpSGHV infection can either be asymptomatic or symptomatic (Abd-Alla *et al.*, 2007a). The asymptomatic state can convert to a symptomatic state, resulting in detectable salivary gland hypertrophy syndrome (SGH). Whereas asymptomatic infection does not pose any immediate danger to the survival and productivity of tsetse colonies, symptomatic infection causes reduction of fecundity (in females), and mating propensity and competitiveness (in males) (Mutika *et al.*, 2012). Therefore, the virus makes it difficult to maintain *G. pallidipes* laboratory colonies, and such colonies have collapsed severally in the recent past (Lietze *et al.*, 2011b).

Tsetse flies devoid of *Wigglesworthia* have low *Wolbachia* and GpSGHV titres, and progenies of such flies are *Sodalis*-free (Wang *et al.*, 2013). In the current study, a series of bioassays were designed to test whether antibiotic-induced immune modulation of microbiome would alter GpSGHV titres and expression of SGH symptoms in the parents and their progenies. In view of delayed effects of artificial GpSGHV infection, all treatments were followed through to the emergence of $F_1$ progenies.

# Materials and Methods

## *Tsetse fly rearing and handling*

All the experiments described in this study were performed on a *G. pallidipes* colony maintained at the Insect Pest Control Laboratory (IPCL), Seibersdorf, Austria. Unless otherwise stated, experimental flies were fed for 15-20 min, 3 times per week, on bovine blood using an *in vitro* membrane feeding system (Feldmann, 1994a). Experimental flies were held in standard round cages (20 cm diameter x 5 cm height) at a density of 75 flies per cage (Male: female ratio of 1: 4), and maintained at a temperature of 23 ± 1 °C, a relative humidity of 75 - 80 % and a 12 h scotophase. Pupae produced from sequential larviposition cycles (hereafter abbreviated with a "G") were collected and incubated at 24°C until adult-eclosion. The progenies were reared under the same insectaria conditions and handled like the parents. All the experiments were replicated at least three times.

## *Preparation of GpSGHV inoculum*

GpSGHV inoculum was prepared from a pair of intact salivary glands dissected from a 10-day old male fly exhibiting overt SGH symptoms as previously described (Kariithi *et al.*, 2013b). The virus titres present in the inoculum was estimated by quantitative polymerase chain reaction (qPCR) as described by Abd-Alla *et al.*, (2009a). By the qPCR method, approximately $1 \times 10^6$ virus genome copies were estimated to be present in a 2 µl aliquot that was used for the inoculations (see below).

## *In vivo GpSGHV replication kinetics*

To monitor GpSGHV replication in the tsetse flies, teneral flies (24 h post adult-eclosion) from the colony were pre-screened for virus infection by PCR amplification of DNA extracted from one intermediate leg as described by Abd-Alla *et al.*, (2007a). Then, six groups of 16 PCR-negative flies (4 males and 12 females in each group) were inoculated with 2 µl of the virus inoculum (dil. 10, 000 times in filter-sterilized PBS) by intra-hemocoelic injection. Immediately after injection, one of the six fly groups was frozen at - 20°C until DNA extraction. The remaining five groups were subjected to one, three, five, seven or nine clean blood meals (every 48 h). For controls, a similar number of flies were mock-infected by filter-sterilized PBS injections. Approximately 48 h post respective blood meals, the flies were frozen at 20°C until DNA extraction.

## *Production and release of GpSGHV particles in salivary secretions*

To investigate the ability of asymptomatic and symptomatic infected flies to produce and release GpSGHV particles via salivary secretions during the *in vitro* membrane

feeding, groups of 24, 10-day-old flies were either mock- or GpSGHV-challenged, and kept in individual holding cells as described above. Subsequently, each of these flies was individually fed on 250 µl clean blood for 28 days post infection (dpi). After the last blood meal, approximately 100 µl of the blood remaining under the feeding membranes was collected and immediately frozen (-20°C). At the end of the 28-days experimental period, flies were also frozen until further analyses.

## *Impact of ampicillin on GpSGHV and microbiome titres*

To investigate the impact of antibiotics on GpSGHV and microbiome titres in parental and subsequent fly progenies, groups of 130 female and 30 male flies were either mock- or virus-infected by intra-hemocoelic injection with 2 µl of PBS or virus inoculum, respectively. The mock- and virus-infected flies were sub-divided into two groups, each consisting of 75 flies; one group was maintained on clean blood meals and the second group on blood amended with 40 µg/ml of penicillin-based antibiotic ampicillin (Sigma). It should be noted that the 40 µg/ml ampicillin concentration was used because it has a negligible negative impact on tsetse productivity (Pais *et al.*, 2008). For controls, two additional fly groups were either mock- or virus-infected as described above, and maintained on non-amended clean blood meals. From all the bioassays, five flies were sampled weekly and mated for ≥ 24 h in small holding cells (3.5 cm diameter x 6 cm height). After mating, males were removed from the fly mating cells and immediately dissected to record incidences of SGH symptoms. The dissected flies were stored at -20°C in individual Eppendorf tubes for later analysis. The females were maintained in the insectaria under the conditions described above until the end of the experimental period (60 days). $F_1$ pupae were collected, placed individually in dated cells and incubated at 24°C until adult eclosion. Newly-eclosed $F_1$ adults were sexed, assessed for incidence of SGH symptoms and preserved as described above. It should be noted that the assessment of the incidences of SGH symptoms in the $F_1$ progenies was performed 24 h post-eclosion.

## *Histology of GpSGHV-challenged parents*

To determine the distribution of GpSGHV and the symbionts in different fly tissues, five female flies were randomly selected from the above-mentioned groups at 37 dpi. It should be noted that the 37 dpi was chosen for sampling of the flies because the virus-challenged flies were noted at that time to be producing $F_1$ progenies exhibiting SGH symptoms. From the selected flies, ovaries, milk glands, and affiliated fat bodies were dissected in Trumps buffer. The tissues were then washed in HEPES-buffered saline (20 mM HEPES, 150 mM NaCl, 1.5 mM $Na_2HPO_4$, pH 7.05), fixed in 4% paraformaldehyde and 1% glutaraldehyde in PBS (pH 7.24) and subsequently processed with the aid of a Pelco BioWave laboratory microwave (Ted Pella, Redding, CA). Tissues were prepared for transmission electron microscopy (TEM) and post-

fixed in 1% $OsO_4$ for 2 h at room temperature. The fixed tissues were washed three times in water, dehydrated in a graded ethanol series (20, 50, 70, and 95 -100%), transferred into 100% acetone, and embedded in Epon-Araldite resin (Electron Microscopy Sciences, EMS, MA). Cured resin blocks were trimmed, thin-sectioned, and collected on formvar-coated nickel 400 mesh grids (EMS). Grids were stained with 2% uranyl acetate and Reynolds lead citrate (Reynolds, 1963). Sections were examined with a Hitachi H-7000 TEM and digital images acquired with a Velveta camera and iTEM software.

## *Quantization of GpSGHV and microbiome titres by qPCR*

Total DNA was extracted from all experimental samples described above using a DNeasy 96 DNA Blood Kit (Qiagen). For quality control of extracted DNA and subsequent data normalization, the tsetse housekeeping *β-tubulin* gene (Caljon *et al.*, 2009) was used in subsequent PCR amplifications. Only those samples positive for *β-tubulin* were further analysed. The primers used for PCR amplifications are listed in **Table 1**.

**Table 1: List of primers used for PCR analyses of GpSGHV and microbiome in *G. pallidipes***: The primers used for quantitative PCR (qPCR) reactions are in bold.

| Target Gene | Primer Name | Primer Sequence (Listed 5'- to -3') | References |
|---|---|---|---|
| *odv-e66* (GpSGHV ORF5) | **qPCRFwd** | **CAAATGATCCGTCGTGGTAGAA** | (Abd-Alla *et al.*, 2009a; 2011a) |
| | **qPCRRev** | **AAGCCGATTATGTCATGGAAGG** | |
| | GpSGHVfwd [b] | GCTTCAGCATATTATTCCGAACATAC | (Abd-Alla *et al.*, 2007a) |
| | GpSGHVrev [b] | GATCCTGCTCGCGTAAACCA | |
| *fliC* (flagellin) (*Sodalis*) | Sod-FliCF [b] | GCAGTTTCAGGATACCC | (Toh *et al.*, 2006) |
| | Sod-FliCR [b] | GGCGGAAAATGGTATAG | |
| | **sodqPCR-FliCF[a]** | **GAAGCCACCGATCCTGTAAC** | (Weiss *et al.*, 2012) |
| | **sodqPCR-FliCR[a]** | **CATCTTTGCCCGTAGAAATCAC** | |
| *thiC* (thiamine biosynthesis) (*Wigglesworthia*) | WiggthiCF [b] | GAACAAGCAATACAAGGTGTAG | (Boucias *et al.*, 2013b) |
| | WiggthiCR [b] | CTATCG GTACATGTCCAGGTCCTTC | |
| | **WiggqPCRthiCF[a]** | **GACATCAAATCGCGTTACTGG** | (Boucias *et al.*, 2013b) |
| | **WiggqPCRthiCR[a]** | **GACTTGTACGTGATATTTCCAAGC** | |
| *β-tubulin* (tsetse fly) | Tsetse-tubulinF | GATGGTCAAGTGCGATCCT | (Caljon *et al.*, 2009) |
| | Tsetse-tubulinR | TGAGAACTCGCCTTCTTCC | |
| *wsp* (*Wolbachia* surface protein) | Wsp fwd | TGGTCCAATAAGTGATGAAGAAACTAGCTA | (Jeyaprakash and Hoy, 2000) |
| | Wsp rev | AAAAATTAAACGCTACTCCAGCTTCTGCAC | |
| *IS* (*Wolbachia* insertion sequence) | ISNew TIR | GGCTTTGTTGCATCGC | (Schneider *et al.*, 2013; Wu *et al.*, 2004) |

GpSGHV titres were quantified by amplification of GpSGHV ORF5 (*odv-e66* gene) (Abd-Alla *et al.*, 2007a; 2009a; 2011a), while *Sodalis* titres were quantified by amplification of the flagellin C (*fliC*) gene (strain *G. m. morsitans*). Attempts to quantify *Wigglesworthia* using the published thiamine biosynthesis protein *(thiC)* gene primers (Weiss *et al.*, 2012) failed; therefore, degenerate primers were designed using the CODEHOP program (Rose *et al.*, 1998), and successfully amplified a partial (645 bp) sequence of *Wigglesworthia thiC* gene (GenBank Accession number KC470073).

Similarly, attempts to detect the presence of *Wolbachia* using the *Wolbachia* surface protein (*wsp*) gene primers (Jeyaprakash and Hoy, 2000), were unsuccessfully. Additionally, *Wolbachia* could not be detected by PCR amplification using a primer (*ISNew*) targeting the flanking terminal inverted repeats (TIR) of the insertion sequence element (Wu *et al.*, 2004). In response to the lack of detectable amplicons for *Wolbachia*, a highly sensitive PCR-blot technique was used as outlined by Schneider *et al.,* (2013). The method is a combination of PCR and hybridization thus allowing for tracing very low *Wolbachia* titres by significantly lowering the detection threshold. A detailed protocol of PCR-blot technique is available in Arthofer *et al.,* (2009).

To prepare qPCR standard curves for both the viral and microbiome' amplicons, regions encompassing each of the target genes were cloned. To estimate equivalent viral and microbiome copy numbers, the PCR-amplified products were purified and quantified using Nanodrop spectrophotometry. Serial dilutions from each standard were run in triplicate to produce a standard curve used to estimate titres in experimental samples (Abd-Alla *et al.*, 2009a). All PCR products were visualized on ethidium bromide-stained 1% agarose gels according to standard protocols.

### *Statistical analyses*

For all the experiments described above, differences in viral and microbiome titres were assessed by analysis of variance (ANOVA). To control the experimental error rate, individual treatments' means were compared with a Tukey - Kramer HSD (Honestly Significant Difference) test (Sokal and Rohlf, 1981). Analysis was performed using Excel® 13 (Microsoft Corp.), RExcel (Baier and Neuwirth, 2007) and R (R Development Core Team, 2010).

## Results

### *Impacts of artificial viral infection in G. pallidipes parents*

Artificial infection of *G. pallidipes* with GpSGHV suspension did not cause any initial detectable perturbations in mating behaviour, feeding and movements of experimental flies. GpSGHV-challenged females produced numbers of $F_1$ fly progenies that mirrored those produced by mock-infected females. Notably, > 95% of the virus-challenged adult parents that were dissected at the end of the 60-day experimental period did not show any detectable SGH symptoms (**Table 2**).

**Table 2: Incidences of SGH symptoms in adult *G. pallidipes* parents from various treatments:** Flies were fed with non-amended or ampicillin-amended blood meals. Groups of adults (4-6) were sampled from the adult parent cages at 6-8 day intervals over a 60 - day experimental period.

| Treatments | | Dissected flies | No. of flies | GpSGHV titres in |
|---|---|---|---|---|
| Blood meal | Infection | (Total #) | exhibiting SGH (%) | flies exhibiting SGH |
| Non-amended | Mock | 70 | 1 (1.43%) | $10^9$ |
| | GpSGHV | 186 | 6 (3.23%) | $10^8$-$10^{11}$ |
| Ampicillin-amended | Mock | 72 | 3 (4.17%) | $10^8$-$10^{11}$ |
| | GpSGHV | 74 | 2 (2.73%) | $10^8$-$10^{11}$ |

At 28 dpi, the virus-challenged flies had significantly higher viral titres (~ $10^{10}$ copies) compared to the titres of ~ $10^4$ copies in flies at 0 dpi ($P < 0.0001$; **Figure 1**).

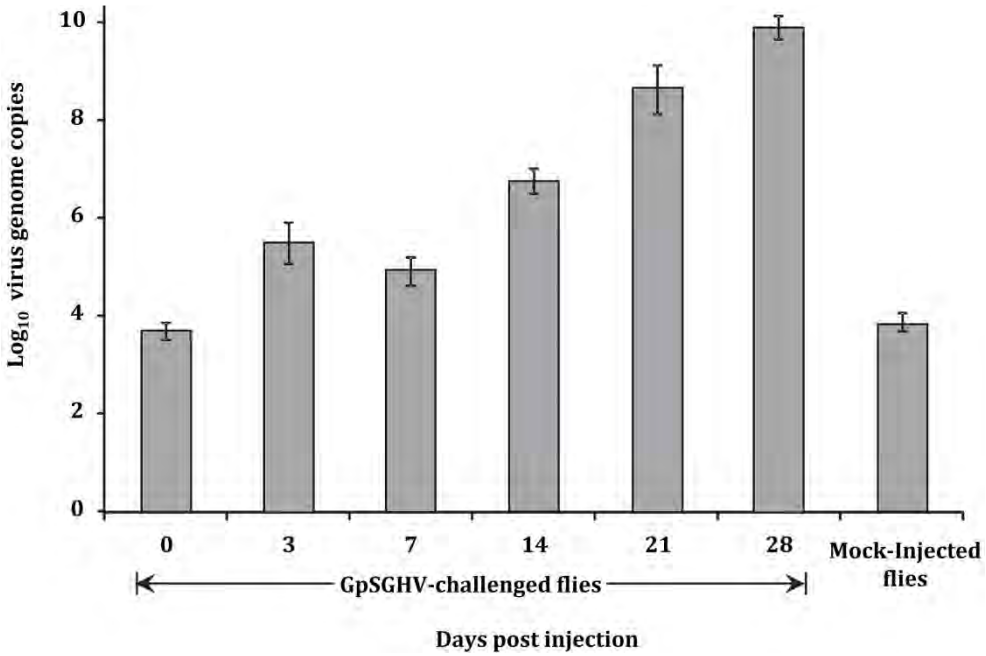


**Figure 1: *In vivo* production of GpSGHV by *G. pallidipes* parents:** Virus titres (represented on the y-axis) were quantified by qPCR on genomic DNA extracted from whole fly bodies. The titters of the virus increased from zero to 28 dpi (x-axis). Error bars are indicated.

Despite the high viral titres, the virus-challenged and mock-infected flies secreted similar amounts of virus copies (~ $10^2$) via saliva into the blood during membrane feeding (**Figure 2**).

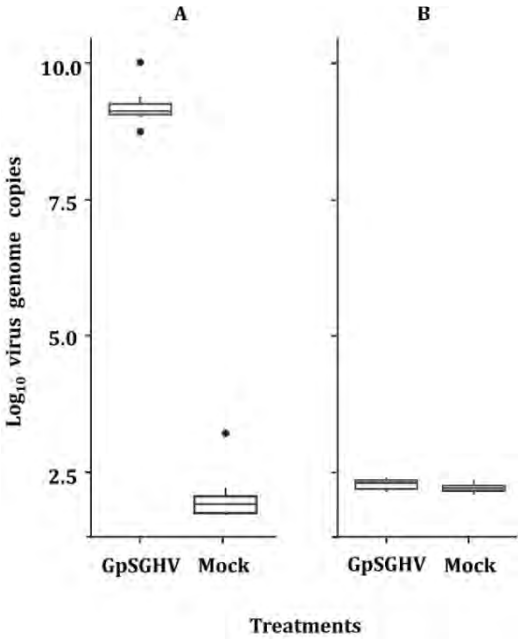


**Figure 2: Analysis of the production and release of GpSGHV particles via salivary secretions:** The graph depicts average numbers of viral genome copies deposited by virus-challenged and mock-infected *G. pallidipes* at 28 dpi. Panel (A) represents viral titres in whole fly bodies, while panel (B) shows the viral titres released via saliva during the *in vitro* membrane feeding. It should be noted that unlike these artificially superinfected flies in this experiment, naturally infected (exhibiting overt SGH symptoms) secrete up to $10^7$ virus copies into the blood during a single feeding event ( Abd-Alla *et al.,* 2010b)

## *Impact of ampicillin on GpSGHV titres in G. pallidipes parents*

Compared to the mock-infected parents, the GpSGHV-challenged parents had significantly higher viral titres, regardless of whether these flies were fed with non-amended or ampicillin-amended blood meals ($P < 0.0001$; **Figure 3**). For the mock-injected parents, the baseline viral titres varied slightly ($\sim 10^{4\text{-}7}$copies) over the experimental period and aging did not correlate to increased viral titres (**Figure 3 A** & **B**). These viral titres agreed well with the virus loads routinely detected in asymptomatic *G. pallidipes* flies under laboratory conditions (Abd-Alla *et al.*, 2009a). For the virus-challenged parents, the viral titres were similar ($10^{8\text{-}11}$): Ampicillin did not significantly alter the viral titres in the parents ($P = 0.598$; **Figure 3 C** & **D**).

Ampicillin-treatment did not cause any initial drastic impacts on the relative duration of pregnancies. Moreover, the size of third instar larvae, length of pupal period, and the adult-eclosion rates of the $F_1$ fly progenies. These fitness parameters were similar for both the mock- and virus-challenged parents (**Table 3**). Compared to mock-injection, GpSGHV-injection led to high mortalities at the end of the 60-day experimental period. The flies fed with ampicillin-amended blood readily mated and produced a normal complement of $F_1$ fly progenies.

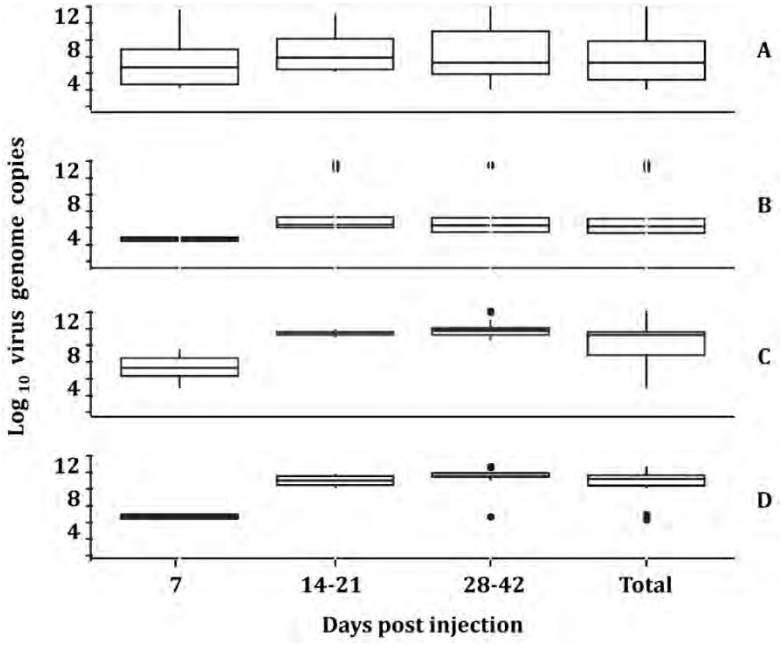


**Figure 3: Impacts of ampicillin on viral titres in *G. pallidipes* parents:** Experimental flies were either mock-injected or GpSGHV-challenged and subsequently treated as follows: Mock-injected flies were fed with either non-amended (A) or ampicillin-amended (B) blood meals. Likewise, GpSGHV-challenged flies were fed non-amended (C) of ampicillin-amended (D) blood meals. Note that the artificial viral infection (C, D) resulted in significant increases in viral levels within 14 dpi. Black dots denote outliers.

As shown in **Table 3**, ampicillin-treatment caused a slight increase in pupal mortalities in the $F_1$ fly progenies produced by both the mock-injected (5.2%) and the virus-challenged adults (7.1%). These mortalities were much less, than the average 20% reduction observed in an earlier study with ampicillin-treatments of *G. m. morsitans* (Pais *et al.*, 2008).

**Table 3: Fitness data on the parental pregnancies and resulting $F_1$ progeny from various treatments:** Flies were fed with non-amended (control), or amended with 40-µg ampicillin/ml blood. Parental mortalities (percentage) at the end of the 60-day experimental period are indicated in column 7.

| Blood meal type | Infection type | Pupae production (for 60 – days) | | | | Parental deaths (%) | Pupation period (days) | Adult eclosion (%) |
|---|---|---|---|---|---|---|---|---|
| | | Day 1-35 | Day 35-60 | Total | Pupal mortality | | | |
| Non-amended | Mock | 238 | 161 | 399 | 18 (4.5%) | 10% | 33-38 | 94% |
| | GpSGHV | 178 | 114 | 296 | 12 (4.1%) | 70% | 32-37 | 95% |
| Ampicillin -amended | Mock | 187 | 199 | 386 | 20 (5.2%) | 11% | 32-36 | 95% |
| | GpSGHV | 179 | 124 | 303 | 22 (7.1%) | 58% | 33-38 | 91% |

## *Impact of antibiotics in the $F_1$ progeny produced by superinfected parents*

Compared to GpSGHV titres of their parents (~ $10^9$ copies per fly; see in **Figure 3**), the viral titres in the $F_1$ fly progenies produced by the ampicillin-treated, virus-challenged parents were similar to the viral titres detected in the $F_1$ progenies produced by the mock-infected parents (1 x $10^4$ copies per fly; **Figure 4**). These virus levels were less than the virus titres (~ 4 x $10^7$ copies per fly) detected in the $F_1$ fly progenies from GpSGHV-challenged parents fed on non-amended blood meals.

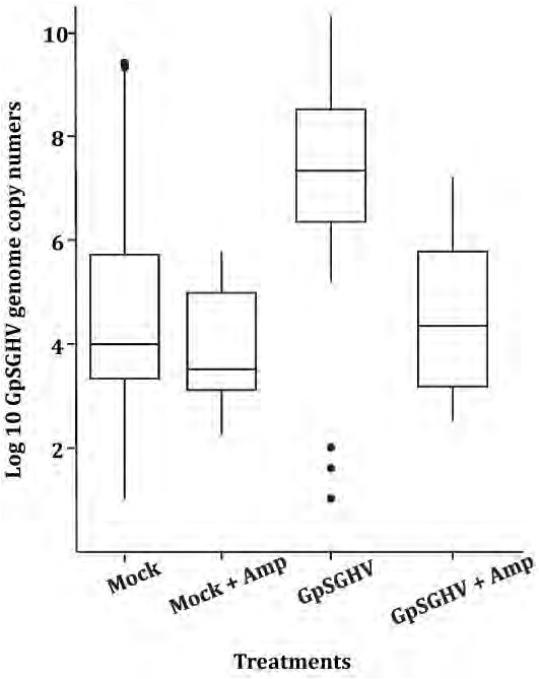


**Figure 4: Analysis of ampicillin-mediated reduction of GpSGHV titres in the $F_1$ progenies produced by the virus-challenged *G. pallidipes* parents:** The viral titres in the $F_1$ progenies produced by the superinfected parents (GpSGHV) were higher than the titres detected in the $F_1$ fly progenies produced by the mock-infected parents (Mock). Feeding the virus-injected parents on ampicillin-amended blood meals (GpSGHV + Amp) reduced the viral titres of their subsequent progenies to levels similar to those detected in the progenies produced by the mock-injected counterparts (Mock + Amp). Black dots denote outliers.

Whereas only 0.7% (2/271) of the $F_1$ fly progeny produced by the mock-injected parents displayed detectable SGH symptoms, 59% of the $F_1$ fly progenies produced by the virus-challenged females exhibited SGH symptoms (**Figure 5 A**). The incidence of SGH symptoms in the $F_1$ fly progenies produced by virus-challenged parents fed on non-amended blood increased from 4.5% in $G_1$-cycle to 100% in $G_4$-cycle (**Figure 5 A**). The ability of virus-challenged females to induce high levels of SGH symptoms in the $F_1$ adults correlated with increases in viral titres in the parental generation: the $F_1$ adults from the $G_3$-cycle were produced by mothers at 28-42 dpi that contained ~ $10^9$ viral copies per fly (refer to **Figure 3 C**). Most notable is the finding that ampicillin-treatment negated the expression of SGH symptoms in the $F_1$ fly progenies produced by the virus-challenged parents (**Figure 5 B**).

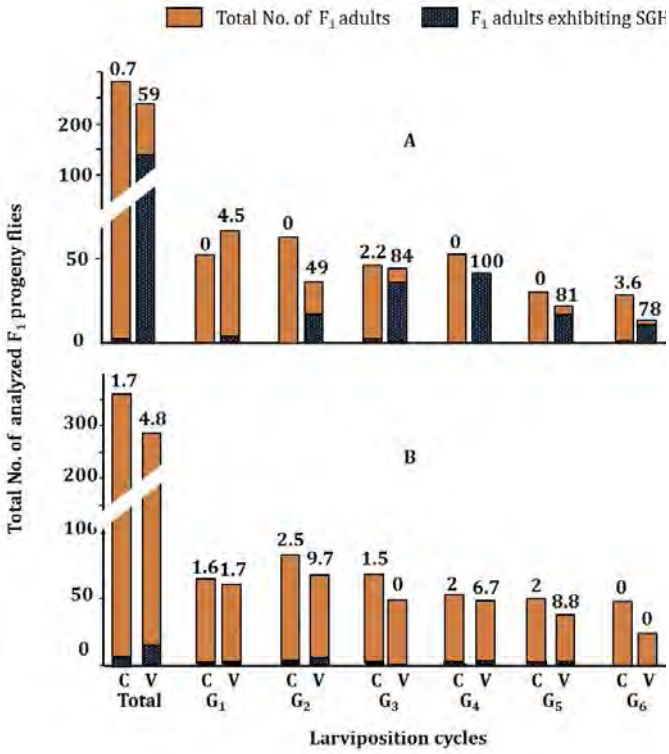


**Figure 5: Analysis of the impact of GpSGHV-challenge in the $F_1$ fly progenies: The n**umber of $F_1$ adults displaying SGH symptoms produced from mock-injected (C), and virus-injected (V) parents fed non-amended (**Panel A**) and ampicillin-amended (**Panel B**) blood meals. Larviposition cycles (G) of the $F_1$ progeny are indicated in the *x*-axis, while the numbers of flies analysed are shown on the *y*-axis. The numbers above the bars represent the percentage of flies exhibiting detectable SGH symptoms. It should be noted that fly dissections to assess SGH were done 24 h post-eclosion of the $F_1$ fly progenies.

Matings were performed on the $F_1$ progenies produced by mock-injected and the virus-injected parents: the most notable outcome of these matings was observed from the $G_4$-cycle onwards. For instance, although matings of the $G_5$-cycle $F_1$ progenies produced by the virus-challenged parents copulated readily, they did not produce any $F_2$ fly progenies. On the other hand, the $G_5$-cycle $F_1$ fly progenies produced by the mock-infected parents produced $F_2$ fly progenies at the normal 10-14 day intervals.

## *GpSGHV transmission to the milk glands via fat body tracheal system*

TEM analysis of the ovaries dissected from the virus-challenged mothers did not provide evidence of GpSGHV replication or the presence of viral particles in the follicle epithelia of the ovaries. Whereas there was no evidence of viral replication in the nuclei of the fat bodies (**Figure 6 A**), viral nucleocapsids were observed aligned along the cytoplasmic face of the nuclear membranes in these tissues (**Figure 6 B**). Clusters of numerous enveloped virions, (localized in vacuoles), were observed in the trachea associated with the fat bodies (**Figure 6 C**). Bacteria, presumed to be *Sodalis* (Balmand *et al.*, 2013), were observed in the regions adjacent to the fat body cells (**Figure 6 D**).

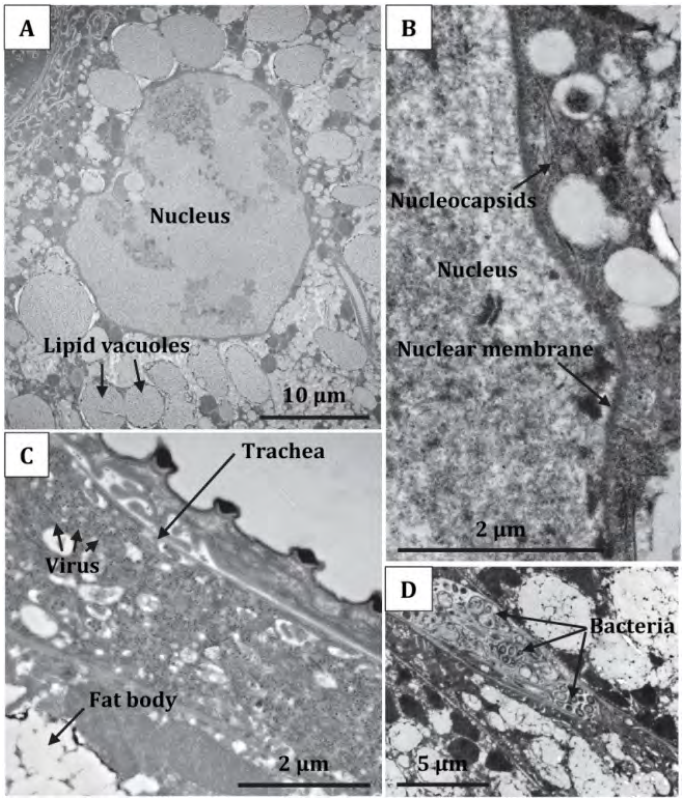


**Figure 6: TEM micrographs of the fat bodies associated with milk glands isolated from GpSGHV-challenged parents at 37 dpi:** (A) fat body cell filled with lipid vacuoles contained nuclei lacking detectable virogenic stroma. In the cytoplasm of fat body cells, numerous nucleocapsids were observed aligned along the nuclear membrane (B). The trachea associated with the fat body tissue contained numerous enveloped and rod shaped virions presumed to be GpSGHV (C), while in regions flanking adjacent cells, there were regions containing bacteria presumed to be *Sodalis* (D).

When observed under TEM, some regions of the milk gland tissues dissected at 37 dpi from the virus-challenged females gave evidence of viral replication (**Figure 7**): in the apical regions of infected milk glands, enlarged multiple nuclei containing numerous nucleocapsids were observed (**Figure 7 A** & **B**). Virions were also observed in tracheal cells associated with the milk glands. Nucleocapsids were observed aligned on the nucleoplasmic face of the nuclear membranes, and bundles of enveloped virions were observed in the cytoplasm (**Figure 7 C**). In multiple TEM micrographs, nuclear membranes remained intact. In the luminal regions of the milk glands, secretory reservoirs associated with viremic cells appeared disintegrated (**Figure 7 D**), some of which contained enveloped virions (**Figure 7 E**). Although virions were also observed to align to the outer membranes enclosing the mitochondria (**Figure 7 F**), there was no evidence whether the virus acquired their envelopes from the mitochondrial membranes. In several sections of the infected gland, there were well-defined regions devoid of cellular organelles, but contained massive numbers of enveloped virions (See **Figure S3**; available online).

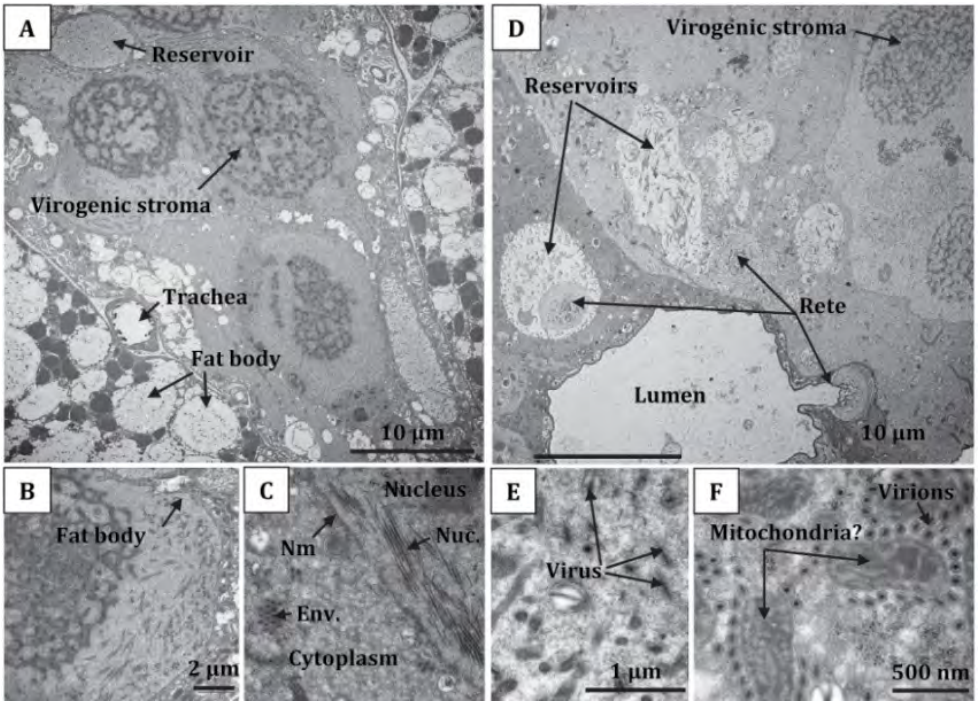


**Figure 7: TEM micrographs of the apical (Panels A, B, and C) and luminal (Panels D, E, and F) regions of GpSGHV-infected milk gland cells of *G. pallidipes*:** (A) A virus-infected milk gland cell surrounded by associated fat body cells and trachea. Multiple enlarged nuclei were observed within the infected gland cells. (B) A high magnification of the virogenic stroma revealing the presence of numerous nucleocapsids. (C) Nucleocapsids were observed to align on the nuclear membrane in certain fields. Bundles of enveloped virus particles (Env.) were observed in the cytoplasm and nucleocapsids (Nuc.) in the nuclei of both virus-infected glands and associated tracheal cells. The nuclear membrane (Nm) remained intact in these viremic cells. (D) Some of the secretory reservoirs were disintegrated, some of which contained enveloped virions (E). In these cells virus particles were aligned to the outer surfaces of mitochondria (F).

## *Presence of Sodalis and Wigglesworthia in colonized G. pallidipes flies*

Comparisons among fly samples from the mock- and GpSGHV-challenged parents revealed that these flies retained similar titres of *Sodalis* and *Wigglesworthia* throughout the lifespan (**Figure 8**). Artificial viral infection had no detectable impact on the symbiont titter in parent flies. In both mock- and GpSGHV-treatments, the titres of Sodalis and Wigglesworthia increased with age from $5 \times 10^3$ (at 7 dpi) to $1 \times 10^5$ (at 21 dpi) cells per fly.

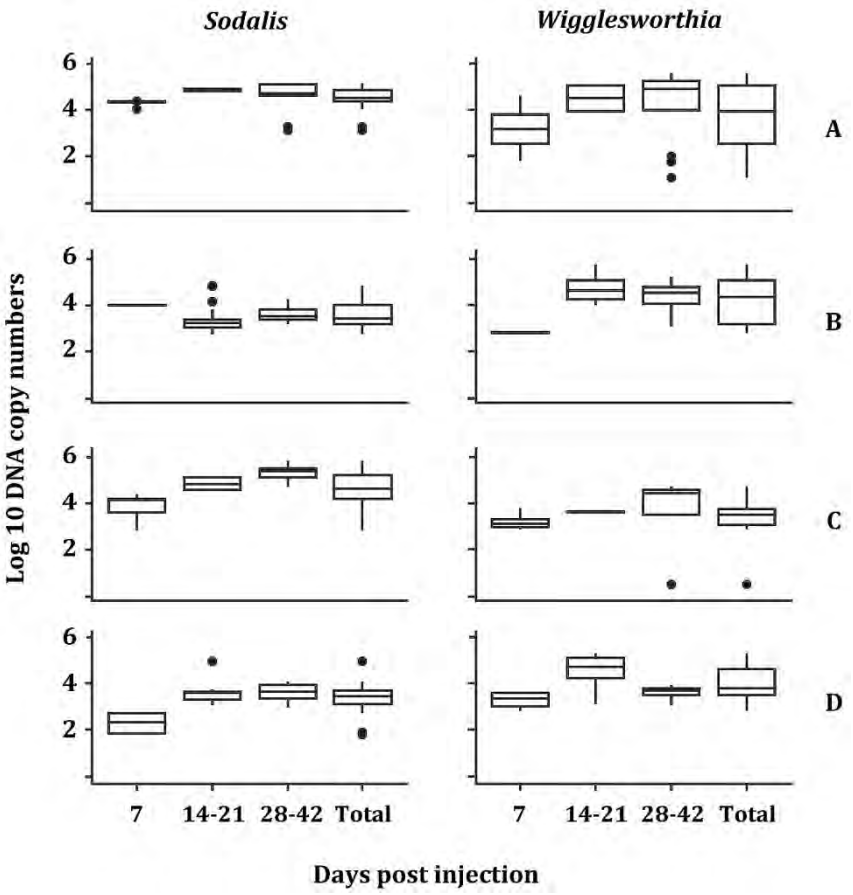


**Figure 8: Impact of GpSGHV-challenge and ampicillin-treatment on *Sodalis* and *Wigglesworthia* titres in *G. pallidipes*:** The relative DNA copy numbers of *Sodalis* and *Wigglesworthia* in the adult parents detected by qPCR on genomic DNA extracted from *G. pallidipes* flies sampled at different intervals post-infection. Mock-injected flies were fed with either non-amended (A) or ampicillin-amended (B) blood meals. Likewise, GpSGHV-challenged flies were fed with non-amended (C) or ampicillin-amended (D) blood meals. The black dots indicate outliers.

Analyses of newly-eclosed $F_1$ fly progenies produced by parents maintained on ampicillin-emended blood revealed that the antibiotic caused significant reduction in the titres of *Sodalis* and *Wigglesworthia* in the fly progenies ($P < 0.0001$; **Figure 9**). It should be noted that the $\sim 10^2$ copies of these symbionts detected in the $F_1$ fly progeny of ampicillin-treatments are close to the qPCR detection limits, suggesting that these $F_1$ fly progenies may be devoid of *Sodalis* and *Wigglesworthia*.

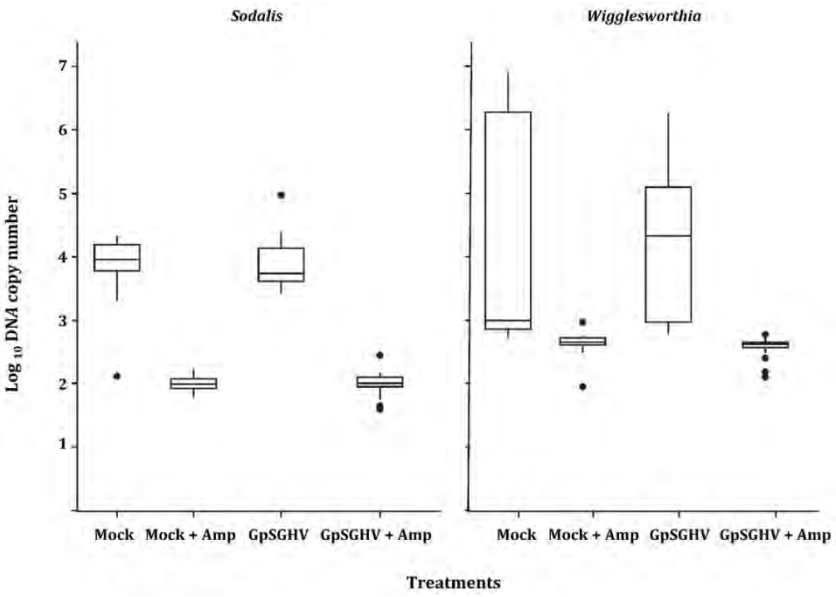


**Figure 9: Titres of *Sodalis* and *Wigglesworthia* in the $F_1$ fly progenies produced by the mock-injected and GpSGHV-injected parents:** The genomic DNA used in the analysis was extracted from flies 24 h post adult eclosion and the titres of *Sodalis* and *Wigglesworthia* quantified by qPCR (see materials and methods section). The relative titres of *Sodalis* and *Wigglesworthia* in the $F_1$ fly progenies produced by mock- and GpSGHV-infected parents fed on non-amended or ampicillin-amended blood meals. Black dots are outliers.

## *Absence of detectable Wolbachia in colonized G. pallidipes flies*

Unsuccessful efforts were made to detect *Wolbachia* in *G. pallidipes* by the highly sensitive blot-PCR technique using primers targeting the single copy *wsp* and the high copy number *IS* genes (Wu *et al.*, 2004) (**Figure 10**). Due to the failure to detect *Wolbachia*, quantification of this symbiont by qPCR was not conducted.

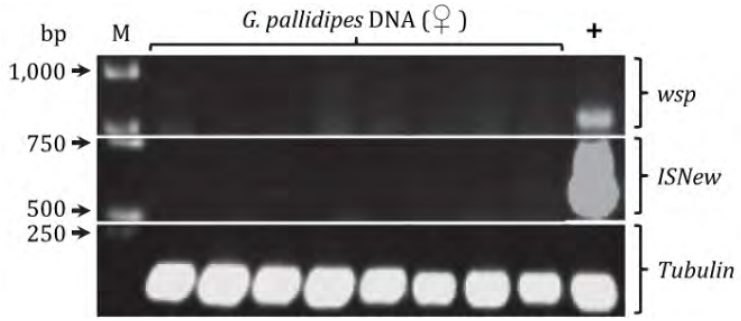


**Figure 10: Absence of detectable *Wolbachia* in *G. pallidipes*:** The presence of *Wolbachia* was tested by primers for the *wsp* and *IS* genes. *Tubulin* was used to assess DNA quality. *G. m. morsitan* mycetome DNA was used as positive control (**+**).

# Discussion

## *GpSGHV persistence in tsetse flies: A tripartite play?*

Theoretically, when a pathogen infects an insect host, three outcomes are possible: the pathogen may either (1) be eliminated by the host's (innate) immune system, or (2) manipulate or escape host's immunity thereby causing virulent effects that kill the host, or (3) may find a balance to co-exist with the host leading to persistent infection. Most likely, the three bacterial endosymbionts of tsetse flies addressed in this chapter fall under the third infection outcomes. Further, in view of the fact that GpSGHV infection is largely asymptomatic (persistent) in tsetse flies, one could argue that the virus also falls into the same category as the bacterial endosymbionts. Here, it is important to keep in mind that similar to the endosymbionts, GpSGHV is vertically transmitted (plus horizontal transmission). Further, the symbionts and GpSGHV share similar (intra-) molecular localization in tsetse flies (Wang *et al.*, 2013; see also **Chapter 8 Figure 2**), implying that the virus and the symbionts may compete for the same host's resources for their survival, transmission and dissemination. One would therefore argue that the tripartite play between the host, the symbionts and the virus might influence the outcome of the infections by for instance regulating the host's gene expression, especially those related to immunity. This argument can be represented as shown in **Figure 11**.

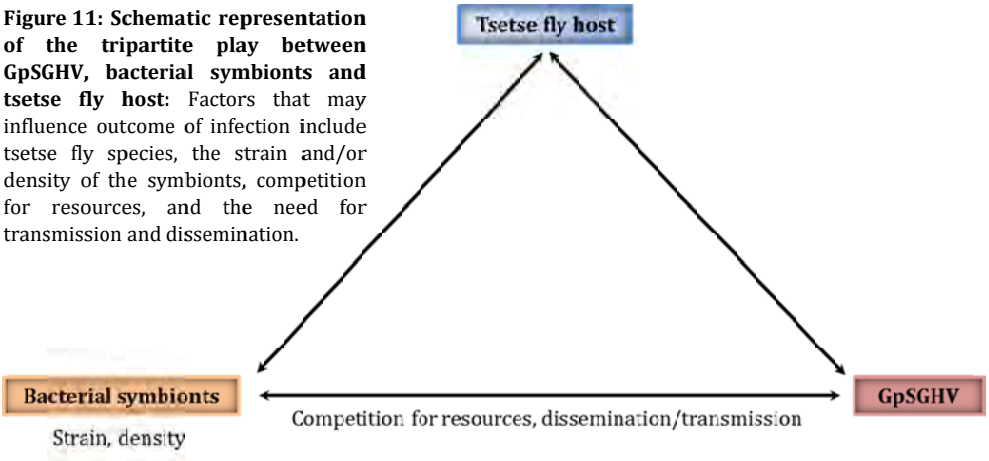


**Figure 11: Schematic representation of the tripartite play between GpSGHV, bacterial symbionts and tsetse fly host**: Factors that may influence outcome of infection include tsetse fly species, the strain and/or density of the symbionts, competition for resources, and the need for transmission and dissemination.

### *GpSGHV-injected G. pallidipes parents do no exhibit SGH symptoms*

From the results obtained in this chapter, it is surprising that, bypassing the cuticle and gut epithelial barriers by intra-hemocoelic GpSGHV-injection did not induce expression of SGH symptoms in the *G. pallidipes* individuals. The variable but low GpSGHV titres in the mock-infected flies suggest that the asymptomatic infection is restricted spatially and temporally to selected cells, or undergoes only partial replication in adult cells to maintain steady-state titres throughout the fly's adulthood. Potentially, the observed changes in GpSGHV titres represent only DNA replication and may not involve production of infectious viral particles.

In contrast to naturally infected *G. pallidipes* colony flies, which secrete large amounts of virus particles via saliva during membrane feeding (Abd-Alla *et al.*, 2010b), the GpSGHV-injected parents secreted negligible amounts of viral particles. This is remarkably different from other studies: GpSGHV-injection into newly larviposited third - instar larvae of *G. m. morsitans* and *G. m. centralis* resulted in expression of SGH symptoms in 100% of the developed adults (Jura *et al.*, 1993; Sang *et al.*, 1997). These earlier reports, in combination with our results on *G. pallidipes* suggest that the injected virus is capable of infecting and replicating during the ontogeny of the salivary gland occurring during pupation. The injected virus appears incapable of either infecting and/or inducing expression of SGH in fully differentiated adult salivary gland cells.

The results obtained from GpSGHV injections into *G. pallidipes* markedly differ from those obtained from injection of the house fly with the *Musca domestica* SGHV (MdSGHV) in several ways. Firstly, unlike in GpSGHV, the house fly-MdSGHV system does not harbour asymptomatic infections, rather, MdSGHV, once present in the host rapidly induces SGH symptoms leading to a massive increase in viral titres within 2-3 dpi (Geden *et al.*, 2011b). Secondly, whereas GpSGHV is not known to replicate in heterologous hosts (except *Glossina* spp.) (Abd-Alla *et al.*, 2011b), injected MdSGHV reaches titres in excess of $10^9$ viral genome copies per fly in the heterologous host, *Stomoxys calcitrans* within 2 dpi, but without onset of SGH symptoms (Geden *et al.*, 2011a). Thirdly, whereas GpSGHV-injection into *G. pallidipes* did not block oogenesis or intra-uterine larval development, very low dose of MdSGHV-injection into *M. domestica* induced a total shutdown of oogenesis and fully developed SGH syndrome in 100% of challenged *Musca domestica* adults (Lietze *et al.*, 2012), and induced sterility in *S. calcitrans* females (Geden *et al.*, 2011a). In *M. domestica*, the increased viral titres could be explained by massive viral replication in the adult salivary gland as early as 3 dpi (Lietze *et al.*, 2009).

## *$F_1$ fly progenies of GpSGHV-injected parents exhibit SGH and are sterile*

Notably, 50%, 84% and 100% of the $F_1$ fly progenies produced by GpSGHV-injected parents in the $G_2$-, $G_3$- and $G_4$-cycles, respectively, exhibited SGH. However, although these $F_1$ adult fly progenies mated readily, they did not produce any $F_2$ progenies. Possibly the males did not successfully deposited sperm into the females' spermathecae (Mutika *et al.*, 2012), or the deposited sperms were not viable. Such virus-induced sterility is believed to be responsible for the collapse of laboratory colonies of *G. pallidipes* (Abd-Alla *et al.*, 2010b), and agrees well with prior reports conducted on symptomatic *G. pallidipes* (Jaenson, 1986) and *G. m. centralis* (Sang *et al.*, 1997). The mating behaviour observed in the *G. pallidipes* in this study is markedly different from the mating behaviour in MdSGHV-infected *M. domestica* females that are refractory to both healthy males and those exhibiting SGH symptoms (Lietze *et al.*, 2007).

## *GpSGHV-injection has negligible impacts on the titres of Sodalis and Wigglesworthia in adult G. pallidipes parents*

Injection of GpSGHV into *G. pallidipes* did not interfere with either the maintenance of *Sodalis* and *Wigglesworthia* in the adult parents, or the subsequent transfer of these bacteria to the $F_1$ fly progenies. The relatively low titres of *Sodalis* detected in the newly emerged $F_1$ adult flies suggest that replication of the free - living *Sodalis* is suppressed during pupation because the symbiont, transferred *in utero* to the developing larva, was incubated for ~ 30 days after larviposition, yet they attained titres of only ≤ $10^4$ cells per fly at adult eclosion. The intracellular *Wigglesworthia* also displayed similar lower titres in teneral $F_1$ adults. It should be noted that the titres of these symbionts detected in the parents in this study were similar to those detected in a similar study in *G. m. morsitans* (Weiss *et al.*, 2008).

## *Ampicillin negates expression of SGH symptoms in the $F_1$ fly progenies*

Most notable of the results of ampicillin-treatment was that this antibiotic blockaded expression of SGH symptoms in the $F_1$ fly progenies. Based on the results of the impacts of ampicillin on the titres of *Sodalis* and *Wigglesworthia*, the lack of detectable SGH symptoms in the $F_1$ fly progenies may reflect on the knockdown of these symbionts from the parents. It can be argued that the removal of these symbionts either suppressed the trans-generational transfer of GpSGHV via the milk glands to the $F_1$, or blocked the ability of the virus to infect and replicate in the salivary gland of the $F_1$ adult progenies.

## *Ampicillin reduces Sodalis and Wigglesworthia titres in $F_1$ fly progenies*

The concentration of ampicillin used in this study did not have dramatic impact on fly fecundity or the rate of $F_1$ adult eclosion. Whereas higher ampicillin concentrations can have detrimental impacts on reproductive capability of tsetse flies (Dale and Welburn 2001), similar low impacts of ampicillin on tsetse productivity have been observed in ampicillin-treated *G. m. morsitans* (Pais *et al.*, 2008; Weiss *et al.*, 2006). Ampicillin did not suppress GpSGHV titres in the $F_1$ fly progenies of the mock-injected parents; virus levels in these individuals were comparable to the $F_1$ fly progenies from mock-infected adults fed on non-amended blood. These findings conflict somewhat with Wang *et al*., (2013), who reported that antibiotic treatment of asymptomatic *G. m. morsitans* resulted in a significant suppression of GpSGHV titres in the $F_1$ and $F_2$ fly progenies. This discrepancy can be explained by the fact that whereas the analyses on *G. m. morsitans* were conducted at 40 - days post – eclosion, the current analyses was conducted on teneral $F_1$ flies (within 24 h post – eclosion).

## *The laboratory stock of G. pallidipes is probably devoid of Wolbachia*

Presence of *Wolbachia* in laboratory stocks of *G. pallidipes* was reported for the first time in 2012 during a large-scale screening on diverse *Glossina* spp. collected from different laboratory and wild fly populations (Doudoumis *et al.*, 2012). Only 1.2% (22/1896) of the analysed *G. pallidipes* samples tested positive for *Wolbachia* infections, majority of which were found in the wild strains. In agreement with the present study, the researchers did not detect any *Wolbachia* infections in the *G. pallidipes* samples collected from the Seibersdorf IPCL-tsetse fly colony. Although GpSGHV was reported in other tsetse species (Doudoumis *et al.*, 2012), the virus has no known harmful impact on *Wolbachia*-harbouring tsetse fly spp. A recent study of *G. fuscipes fuscipes* populations suggested that the titres of PCR-detectable GpSGHV are influenced by the tsetse fly genotype, and is inversely correlated with the prevalence of *Wolbachia* (Alam *et al.*, 2012). It has also been reported that *Wolbachia* is absent from some of the major economically important mosquitoes such as *Anopheles* spp. (Hughes *et al.*, 2011). Studies have demonstrated that while none of the *Wolbachia*-infected *Aedes aegypti* tested positive for Dengue virus (DENV) after oral infection, 30%-100% of *Wolbachia*-free mosquitoes were DENV-infected (Moreira *et al.*, 2009). In *Ae. Albopictus*, *Wolbachia* can block block transmission of dengue and chikungunya viruses, but without concomitant elevation of *Drosophila*'s immune genes, suggesting that strong *Wolbachia*-mediated viral inhibition operates independent of the host's immune pathways. In view of the recently published report, demonstrating the antiviral activity induced by *Wolbachia* in infected tsetse fly hosts (Wang *et al.*, 2013), the possible absence of *Wolbachia* in the *G. pallidipes* colony may explain the severe negative impact of GpSGHV on large-scale *G. pallidipes* colonies.

## *The fat body tracheal system may provide a conduit for trans-generational transmission of GpSGHV in G. pallidipes*

Evidently, ampicillin was unable to access and/or kill the intracellular phenotypes of *Wigglesworthia* and *Sodalis*. This is to be expected because, on the one hand, *Wigglesworthia* exists in two physiologically distinct phenotypes: a bacteriome-form for dietary supplementation (in mid-gut), and a milk gland-form which is transmitted to progeny (Pais *et al.*, 2008). On the other hand, *Sodalis* resides intra - and extra – cellular in the midguts, and is detectable in the fly hemolymph (Cheng and Aksoy, 1999). Since both *Wigglesworthia* and *Sodalis* are transmitted to the intrauterine progeny through milk gland secretions (Pais *et al.*, 2008), the clearance of the extracellular populations of these symbionts by ampicillin-treatment explains the absence of these bacteria in the $F_1$ progeny. The ampicillin-treatment and the concomitant reduction of *Wigglesworthia* and *Sodalis* titres in the $F_1$ fly progenies of GpSGHV-challenged parents correlated well with suppression of the expression of SGH symptoms in the $F_1$ fly progeny.

This result was surprising because microbial symbionts have been reported to prime the insect immune system, thereby increasing host resistance to infection by various viruses, protozoans, and nematodes (Haine, 2008; Moreira *et al.*, 2009; Rances *et al.*, 2012; Teixeira *et al.*, 2009). In tsetse flies, the bacterial associates modulate host innate defences at several levels. For instance, *Wigglesworthia* stimulates production of the catalytically active peptidoglycan recognition protein (PGRP-LB) in the midgut bacteriome of *G. m. morsitans* (Wang *et al.*, 2009). This enzyme scavenges the peptidoglycan fragments produced by *Wigglesworthia* preventing activation of the innate defence systems that can influence tsetse fly fitness and reduce fecundity. An important outcome is that antibiotic-treated parents produce $F_1$ fly progenies that are highly sensitive to trypanosome infection (Pais *et al.*, 2008). However, in the case of *G. pallidipes,* suppression of the symbionts did not influence GpSGHV titres in the parental generations, suggesting that the virus is unresponsive to ampicillin-induced alterations of the innate defence system. Logically, one would expect that the reduced titres of *Sodalis*, *Wigglesworthia*, plus the absence of *Wolbachia* in the $F_1$ progenies (resulting from the blockade of the maternal transmission of these symbionts from the parents) would make these progenies more prone to development of SGH.

## *Microbiome modulate viral-host interactions*

Microbiome is actively involved in modulation of virus-host interactions (Ishikawa, 1989). For instance, aphids treated with tetracycline, presumably killing the symbiont *Buchnera* sp., were unable to transmit potato leaf roll luteovirus (PLRV), and resulted in loss of capsid integrity of the virions (van den Heuvel *et al.*, 1994). It has been suggested that symbiont-encoded chaperonins such as GroEL (also known as Hsp 60)

facilitate viral trafficking in aphids (Filichkin *et al.*, 1997; Hogenhout *et al.*, 2000). The GroEL-virus interaction is required to retard proteolysis, and is essential for virus retention in the aphid hemolymph on their way to the salivary glands (van den Heuvel *et al.*, 1997). Similarly, feeding the whitefly *Bemisia tabaci* with anti –*Buchnera* GroEL antiserum caused > 80% reduction in tomato yellow leaf curl virus (TYLCV) transmission, and reduced hemolymph viral titres to amounts below the threshold detection by Southern blot hybridization (Morin *et al.*, 1999). This reduced transmission of TYLV has recently been shown to result from an inability of the virus to cross the gut/hemolymph barrier (Morin *et al.*, 2000). More recently, Gottlieb *et al*., (2010) demonstrated the GroEL encoded by the endosymbiont *Hamiltonella defensa* is responsible for the efficient TYLCV transmission by the B - biotype whitefly. On the other hand, the Q-biotype, which harbours *Wolbachia* and lacking *H. defensa*, is a less efficient TYLCV transmitter.

In tsetse flies, establishment of infection in the midgut and subsequent transmission of trypanosomes has been reported to be mediated by a GroEL-like chaperonin encoded by *Wigglesworthia* and *Sodalis* (Haines *et al.*, 2002). Whereas little is known about the distribution of the *Sodalis* GroEL, the *Wigglesworthia* GroEL is localized within the tsetse bacteriome, and is the most highly expressed midgut protein. Notably, proteomic analysis of GpSGHV revealed that the viral envelope, in addition to containing peptides associated with viral proteins, contained many peptides that displayed homology to the host and symbionts proteins. Significantly, a series of *Sodalis* proteins including the major outer membrane lipoprotein, outer membrane protein A, outer membrane protein F, hypothetical phage protein, peptidoglycan-associated lipoprotein, type-III secretion apparatus, and the GroEL (also known as Hsp 60) peptide were detected in the envelope fraction (Refer to **chapter 4**). The selective sequestration of these bacterial peptides in the viral envelope suggests a functional role in the initial establishment of midgut infection, immune evasion and transport of virions to the salivary glands via hemolymph. Antibiotic treatment would, most likely, reduce the levels of bacterial products available for decorating the viral envelope.

## Concluding remarks and future perspectives

Whereas injection of GpSGHV in *G. pallidipes* did not induce expression of SGH symptoms in adult flies, it increased viral titres in the milk gland tissues, eventually leading to expression of SGH in the $F_1$ fly progenies (0-3% in $G_1$, 10-30% in $G_2$, 40-60% in $G_3$, and 100% in $G_4$ onwards). Ampicillin-treatment suppressed symbionts' titres, and blocked trans-generational virus transmission to the $F_1$ fly progenies. These findings suggest that GpSGHV has evolved in close association with the microbiome. This study raises several issues that need further investigation: (1) The replicative pathways of GpSGHV during asymptomatic infection need to be investigated. (2) It is

important to determine which viral genes are specifically expressed during asymptomatic infection in the presence or absence of the microbiome and/or other associated microorganisms such as trypanosomes. (3) It should be investigated whether the lack of *Wolbachia* in the laboratory *G. pallidipes* colonies is truly linked to maintenance of the asymptomatic or persistent GpSGHV infections. (4) To provide a more comprehensive understanding of the determinants of the outcome of host-symbiont-GpSGHV infections, it is important to elucidate the interactions that underpin tsetse fly's immune function, GpSGHV infection and the bacterial symbionts. (4) The role of tsetse fly's hemolymph/haemocytes in the establishment, transmission and dissemination of GpSGHV should be further investigated.

## Acknowledgements

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# Chapter 7

# Management of GpSGHV infections in *G. pallidipes* colonies[6]


## Abstract

In the laboratory colonies of the tsetse fly, *Glossina pallidipes* (Diptera; Glossinidae), titres of *G. pallidipes* salivary gland hypertrophy virus (GpSGHV) higher than $10^9$ genome copies per fly lead to expression of the salivary gland hypertrophy symptoms (SGH). Expression of SGH is accompanied by a decline in the life span and productivity of the flies. Flies with such high viral titres release large amounts of virus particles ($\sim 10^7$ copies per fly) via saliva secretions during a single feeding event in the *in vitro* membrane feeding system used in the colonies. Since the released viral particles are infectious *per os* to healthy flies, this feeding regime favours efficient GpSGHV transmission as each membrane may be used to feed up to ten sets of fly cages in succession. Various approaches were instigated to develop cost-effective GpSGHV management strategies, with the overall goal to obtain either virus - free colonies, or at least reduce the viral titres to levels that do not compromise colony productivity and survival. This chapter describes an essential advancement in the management of GpSGHV in *G. pallidipes* colonies, by modification of the *in vitro* membrane-feeding regime. After 28 months of implementation of the modified feeding regime, GpSGHV titres were not only successfully maintained at a remarkably low level ($\sim 10^{2.5}$ copies), but also the expression of SGH symptoms was eliminated from the colony. The developed feeding regime - at equal costs with the traditional feeding regime – will greatly increase the productivity of *G. pallidipes* colonies, which are crucial for programmes to control African trypanosomoses and for future research. The findings are also of wider interest to those concerned with cultures of other insects.


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# Introduction

For eradication of tsetse fly (Glossina; Diptera) and trypanosomosis in sub-Sahara Africa, various large-scale colonies of the tsetse fly, *Glossina pallidipes* have been established. One of such colony was initiated at the Joint FAO/IAEA Insect Pest Control Laboratories (IPCL), Seibersdorf, Austria, and another colony at the Kality Tsetse Rearing and Irradiation Centre (Kality Centre), Ethiopia. The principle purpose of these colonies was *en masse* production of sterile males for the sterile insect technique (SIT) programs, mainly in the South Rift Valley of Ethiopia, where *G. pallidipes* is the species targeted for eradication. However, maintenance of productive colonies proved difficult due to deleterious effects of high infection rates by the *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV) (Abd-Alla *et al.*, 2010b; 2011b).

Among the tactics considered to salvage tsetse colonies was the initiation of a new, GpSGHV-free colony by obtaining fresh materials using pupae and/or immature females collected from the target wild tsetse populations. The materials would then be screened for GpSGHV infections by PCR (Abd-Alla *et al.*, 2007a). However, initiating new tsetse colonies is difficult because field-collected pupae are usually undersized and have low yield, while the longevity and productivity of such immature females are usually too low to allow considerable increase in colony sizes (Nash, 1969). Field-collected materials are also likely to harbour covert GpSGHV infections that may escape the screening. This approach is labour-intensive and was therefore deemed untenable, and thus driving us to search for other more cost-effective approaches for GpSGHV control strategies. To enable development of cost-effective GpSGHV control scheme(s), studies were performed to investigate: (i) GpSGHV epidemiology in *G. pallidipes* (Abd-Alla *et al.*, 2010b; Kariithi *et al.*, 2013a), (ii) GpSGHV proteome and virion components (Kariithi *et al.*, 2013b; Kariithi *et al.*, 2010), and (iii) potential roles played by viral and host proteins in establishment of infections (Kariithi *et al.*, 2011). The data generated from these investigations would subsequently be applied to develop an integrated strategy to manage GpSGHV infections in the mass production of tsetse flies, especially for the *G. pallidipes* colonies.

Since the *in vitro* membrane feeding regime used in tsetse mass-production facilities (Feldmann, 1994a) significantly augments GpSGHV transmission – because each membrane is used to feed up to ten sets of fly cages in succession (Abd-Alla *et al.*, 2010b) – it was conceptualized that modification of this feeding regime could block horizontal GpSGHV transmission. This chapter details on successful elimination of the salivary gland hypertrophy syndrome (SGH) caused by GpSGHV infections, and on the reduction of GpSGHV infections in the laboratory *G. pallidipes* colonies through implementation of a modified feeding regime without additional resources.

# Materials and methods

## *Tsetse fly colonies*

Unless otherwise stated, the experiments described in this chapter were performed on five different *G. pallidipes* colonies, (including the two fly colonies mentioned above). The first three colonies originated from pupae collected near Tororo, Uganda, and were subsequently adapted to laboratory conditions at; (1) the IPCL, Seibersdorf, Austria (hereafter referred to as "Seibersdorf Tororo" colony), or (2) the Institute of Zoology, Slovak Academy of Sciences, Bratislava, Slovakia (hereafter referred to as "Bratislava Tororo" colony), and (3) the Kality Centre, Ethiopia (hereafter referred to as "Kality Tororo" colony). The other two colonies were derived from pupae collected near Arba-Minch, Ethiopia, and adapted to laboratory conditions at Kality Centre during (4) the period 1999 - 2001 (hereafter referred to as "Kality Arba-Minch old" colony), and (5) the period 2008 - 2010 (hereafter referred to as "Kality Arba-Minch new" colony). With the exception of fly dissections, all sample analyses were performed at IPCL. Samples from the other four colonies were shipped to IPCL for subsequent analyses.

## *Colony maintenance, handling and feeding regimes*

Experimental flies were kept in standard round holding cages (diameter of 20 cm and height of 5 cm) at a fly density of 75 flies per cage, with netting on top and bottom for feeding and collection of larvae, respectively (Vreysen, 2001). Colony flies were maintained at a temperature of 23 ± 1 °C, a relative humidity of 75 - 80 % and a 12 h scotophase. Fly mating was done at a 1: 4 (male: female) ratio, in a self - stocking system whereby, colonies are usually composed of weekly fly units. Each unit was established by pupae collected at the end of each week followed by incubation at 24 °C until adult - eclosion (emergence of teneral flies). The newly-eclosed adults were then used to supply the colony with parents for subsequent fly generation (**Figure 1**).

The flies were offered meals of fresh-frozen defibrinated bovine blood 3 times per week for 10 – 15 min per feeding event, using an *in vitro* feeding system consisting of textured anodized aluminium feeding trays (measuring 480 x 480 x 10 mm) and silicone membranes (Feldmann, 1994a). The blood was obtained from Svaman spol s.r.o., Myjava, 90701, Slovakia. The performance of the colonies was monitored in terms of fecundity (pupae productivity per female), and mortality (checked weekly). All pupae from the colonies described above were collected each week, and used to compose new colony units as described below.

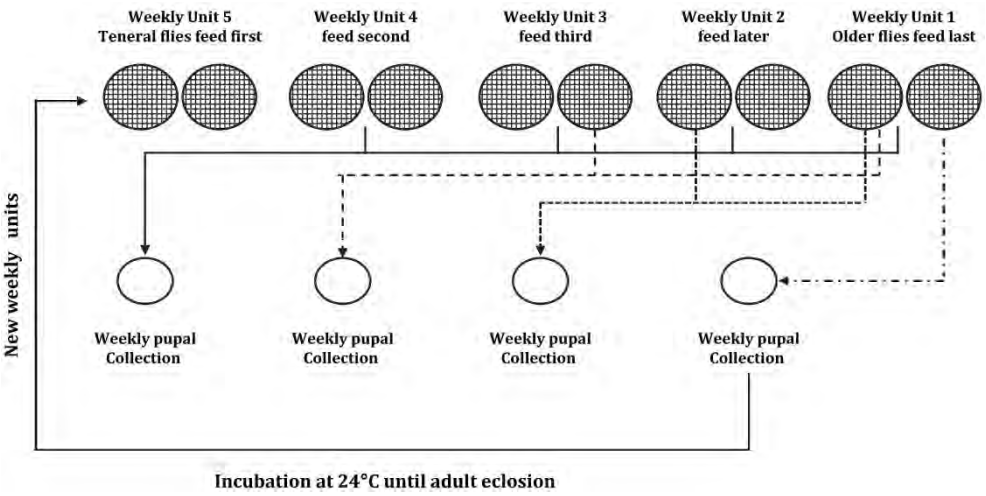


**Figure 1: - Schematic representation of self-stocking system practiced in the mass production of *G. pallidipes*:** Pupae are collected weekly, incubated at 24 °C until emergence of teneral flies. The newly-eclosed adults supply parents for subsequent fly generation.

Two fly feeding regimes were used: The first was the standard membrane feeding regime usually practiced in large-scale tsetse production facilities (Feldmann, 1994a), whereby up to 10 fly-holding cages are offered blood meals on the same membrane-covered blood trays in succession. The second system was a "clean feeding" regime (hereafter denoted as "clean feeding"), whereby each fly-holding cage was provided with fresh blood at each meal.

## *Development and implementation of clean feeding regime*

The feasibility of implementation of the clean feeding was piloted at the Seibersdorf Tororo colony. For this, teneral (newly-eclosed, non-fed) flies were randomly selected and placed into fly holding cages. Flies were subsequently offered a clean blood meal and thereafter, these flies and their subsequent fly progenies were always the first to feed on fresh blood during the entire experimental period. This colony, denoted as "clean feeding colony 1" (hereafter abbreviated as "CFC - 1"), was subsequently expanded by addition of teneral flies emerging from CFC - 1 parents until the maximum number of cages ($n$ = 48) was attained that could be fed first (during one round of feeding) on feeding trays and silicon membranes available at IPCL. Subsequently, when the 48 fly cages for CFC - 1 were attained, surplus flies from CFC - 1 progeny were fed on the same membrane in a second feeding round after feeding CFC - 1. This second-round fed group of flies, denoted as "clean feeding colony 2" (abbreviated hereafter as "CFC - 2"), was always maintained in the second feeding round after feeding CFC - 1 throughout the entire experimental period. During the

institution of CFC - 1 and CFC - 2, the main (regular) colony at Seibersdorf was always fed on the same membranes used to feed CFC - 1 and CFC - 2 (at the third and subsequent feeding rounds). In the course of the experimental period, this regular (Seibersdorf Tororo) colony at IPCL, was gradually replaced by a colony denoted as "normal feeding colony", and is hereafter abbreviated as "NFC" (see **fig 2).**

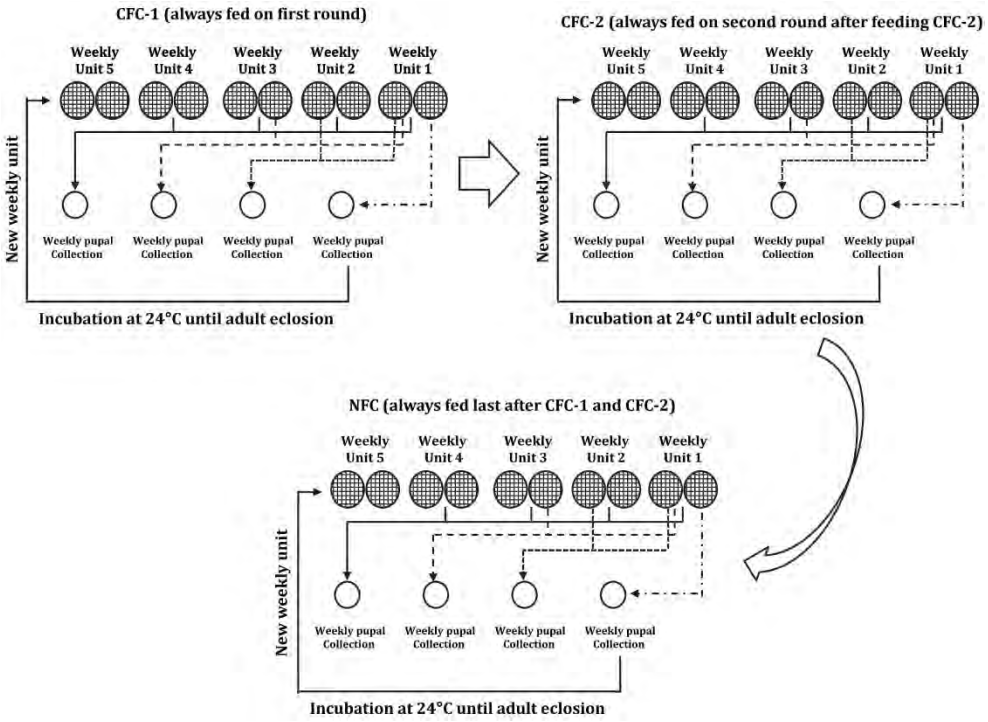


**Figure 2: - Schematic representation of self-stocking system of the proposed clean feeding regime:** - Establishment and maintenance of CFC - 1, CFC - 2 and NFC colonies of *G. pallidipes* colonies at the IPCL, Seibersdorf, Austria, over a period of 28 months.

## *Impact of clean feeding regime on GpSGHV infections*

To determine the impact of clean feeding on GpSGHV titres and SGH prevalence, the initial viral infection status in the Seibersdorf Tororo colony that was used to initiate the clean feeding system was compared with the infection status of the CFC - 1, CFC - 2 and the NFC. To monitor SGH prevalence, a total of 1, 326 flies were randomly selected from the five colonies and the CFC – 1, CFC – 2, and NFC outlined above. Sampling was performed over a period of 28 months (2010 – 2012), and consisted of males and females of different ages, different weekly colony units, and 2 - 110 days post adult eclosion (fly emergence). For each sampling, 20 flies were collected from

each unit. The flies were briefly (3 - 5 min) anesthetized at – 20 °C, placed on ice and immediately dissected to assess the status of the salivary glands. To determine GpSGHV titres, 8 - 16 flies were randomly selected as described above for determination of occurrence of SGH. Total DNA was extracted from the sampled individual flies using the DNeasy kit (Qiagen) according to the supplier's instructions. Quantitative PCR (qPCR) was carried out on extracted DNA using primers and conditions previously described (Abd-Alla *et al.*, 2009a).

### *Statistical analysis*

Statistical analyses for all the experiments described above were assessed by analysis of variance (ANOVA), a Tukey - Kramer HSD (Honestly Significant Difference) test (Sokal and Rohlf, 1981) and Excel® 13 (Microsoft Corp.), RExcel (Baier and Neuwirth, 2007).

## Results

### *SGH Prevalence in the different G. pallidipes colonies*

Of the 1,326 flies sampled from the five colonies used in this chapter, an average of 19.15 % ($n$ = 254) had SGH (**Table 1**). Of the five *G. pallidipes* colonies, the two colonies maintained at Kality Centre showed higher SGH prevalence compared to the other colonies; the Kality Arba-Minch-old colony having the highest prevalence (73.8 %). The SGH prevalence values shown in Table 1 are based on fly dissections, implying that some flies that may not have developed full SGH may have been missed.

**Table 1: Prevalence of GpSGHV in different *G. pallidipes* colonies:** Numbers of flies randomly selected from five different *G. pallidipes* colonies to assess SGH prevalence.

| Colony No. | Name of colony | Total no. of dissected flies | No. of flies exhibiting SGH | % of flies exhibiting SGH |
|---|---|---|---|---|
| 1 | Seibersdorf Tororo | 244 | 9 | 3.69 [b, c] |
| 2 | Bratislava Tororo | 309 | 9 | 2.91 [c] |
| 3 | Kality Tororo | 322 | 32 | 8.77 [b] |
| 4 | Kality Arba-Minch Old | 266 | 195 | 73.84 [a] |
| 5 | Kality Arba-Minch New | 185 | 9 | 4.45 [b, c] |
| **Total** | | **1,326** | **254** | **19.15** |

[a, b, c]: Indicate the significance of differences in SGH prevalence between colonies. Colonies with same letter (e.g. "b" and "c" for Seibersdorf Tororo and Kality Arba-Minch colonies) had insignificant differences in SGH prevalence, as opposed to colonies with different letters.

SGH prevalence varied significantly ($P < 0.00001$) between the five *G. pallidipes* colonies (**Figure 3**). No significant differences were observed in SGH prevalence between the Seibersdorf Tororo and Kality Arba-Minch New colonies ($P = 0.087$). Although the Seibersdorf Tororo, Bratislava Tororo and Kality Tororo colonies had

same origin, SGH prevalence was comparatively higher in the Kality Tororo colony. Taken together, it can be speculated that the observed variations in the prevalence of SGH within and between tsetse colonies may depend on environmental and/or genetic factors other than the age of the flies.

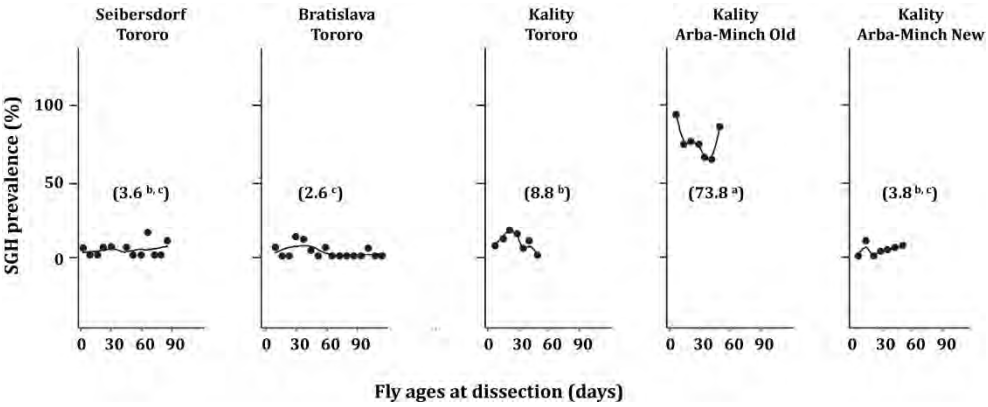


**Figure 3: - Prevalence of SGH in the five *G. pallidipes* colonies analysed in this chapter:** The flies were randomly selected at different time points from the different colonies and dissected to assess SGH. The numbers in the parentheses represent the average SGH prevalence (percentage) for each of the colonies. The line is the smoothed regression.

## *Impact of clean feeding regime on GpSGHV infections*

Fly dissections revealed that SGH prevalence in the CFC - 1 colony was significantly reduced over the experimental period (**Figure 4**). Within the first 6 months, SGH prevalence was reduced from the documented average SGH prevalence of ~ 10% for regular *G. pallidipes* colonies to an average of 2.9 %, irrespective of fly ages at the time of dissections. After 11 months, SGH prevalence in the colony was further reduced to 2.3 %, and SGH symptoms were eliminated after 28 months. Within the same experimental period, a similar trend was observed in the CFC - 2 colony.

In the NFC colony, there was a transitory raise in SGH prevalence from 10 to 24 % during the first 6 months. Although there was no significant change in SGH prevalence (~ 23.7 %) after 11 months in NFC, younger flies (0 - 20 days old) showed a higher prevalence of ~ 50 %. However, after 28 months, SGH prevalence was significantly reduced to 3.6 %. Whereas SGH prevalence in the NFC appeared to fluctuate with fly ages during the first year, the prevalence stabilized later irrespective of the age of the flies (see **Figure 3**). Possibly, the initial rise in SGH prevalence was due to teneral flies of NFC being exposed to GpSGHV circulating in the CFC - 1 and CFC - 2 feeding before the viral titres in these two colonies dropped to a low levels.

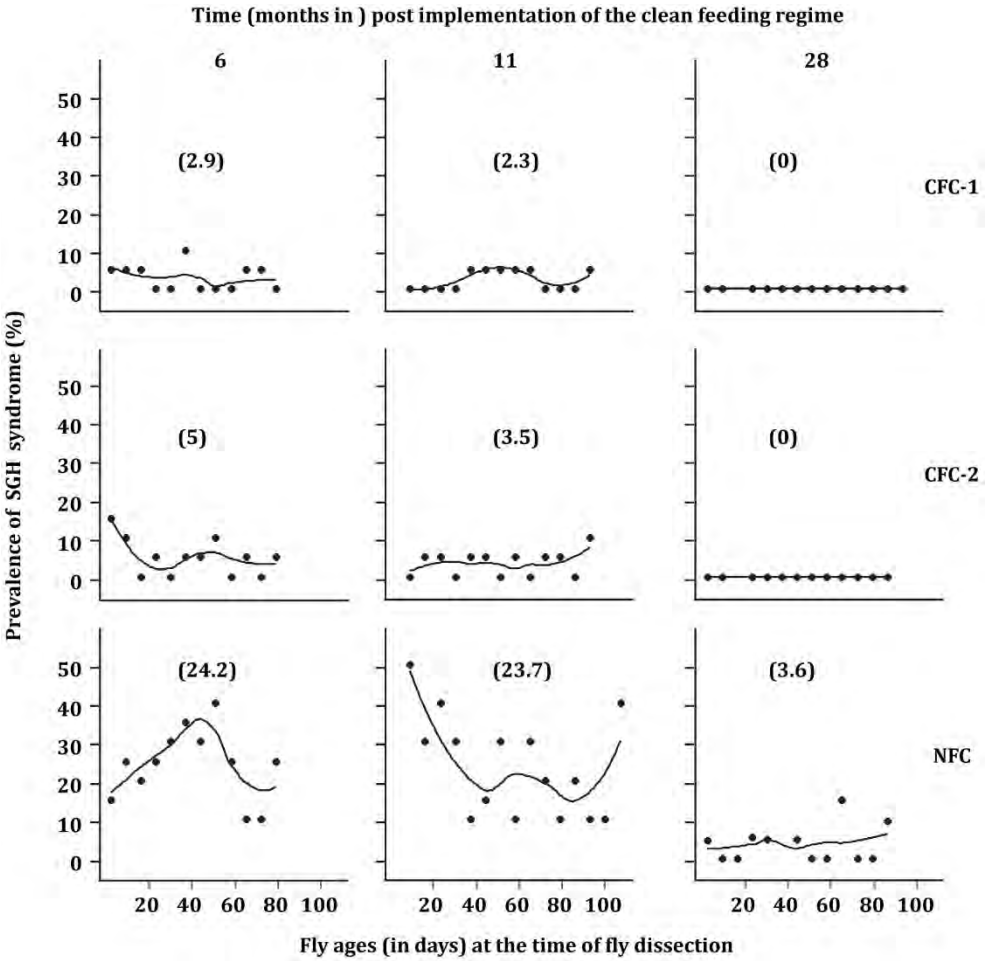


**Figure 4: - Effect of long – term clean feeding regime on SGH prevalence:** Flies sampled from CFC - 1, CFC – 2 and NFC colonies were randomly selected at 6, 11 and 28 months after implementation of the clean feeding system, and dissected to assess SGH. The numbers in the parentheses represent the average SGH prevalence (percentage) for each of the colonies.

At the end of the 28 - months' experimental period, GpSGHV titres in CFC - 1, CFC - 2 and NFC colonies were significantly lower ($\sim 10^{2.4}$, $10^{2.7}$ and $10^{3.3}$ virus genome copies per fly, respectively) compared to the titres ($\sim 10^{7.4}$ copies) in the main (Seibersdorf Tororo) colony that was used to initiate the clean feeding regime ($P < 0.0001$) (**Figure 5**).

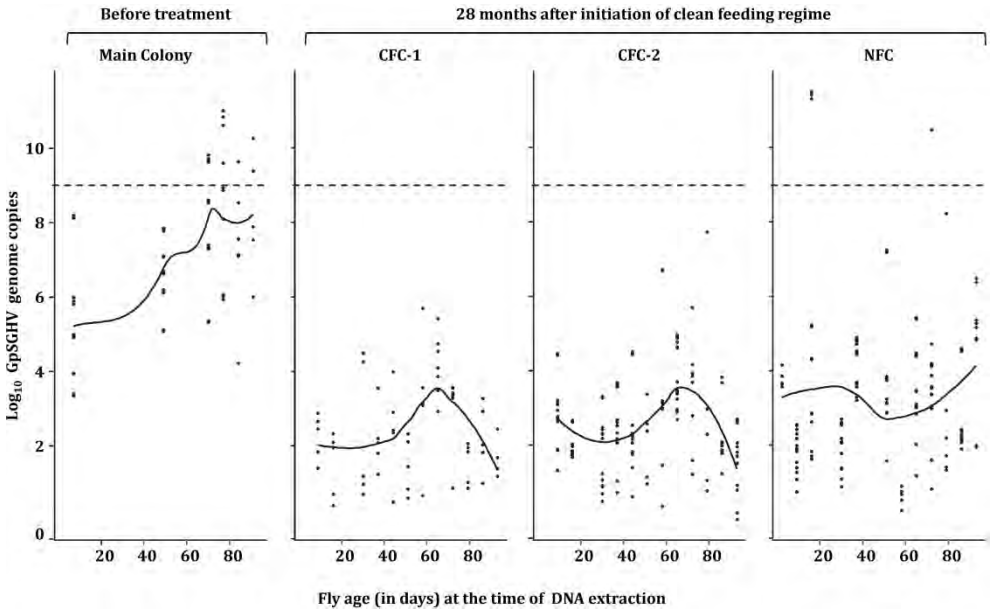


**Figure 5: - Effect of clean feeding regime on GpSGHV titres:** GpSGHV titres were determined by qPCR of the main (Seibersdorf Tororo) colony before initiation of the clean feeding regime, and from CFC - 1, CFC - 2 and NFC after 28 - months' of implementation of the clean feeding regime. The dotted (---) line represents the threshold which correlates with GpSGHV titres ($10^9$) responsible for development of SGH syndrome (Abd-Alla *et al.,* 2009a).

Whereas GpSGHV titres in the main (Seibersdorf Tororo) colony increased significantly ($P < 0.0001$) with increase in fly ages, there was no apparent correlation between viral titres and fly ages in CFC - 1 and CFC - 2. Rather, in the CFC - 1 and CFC - 2, the viral titres decreased in flies aged ≥ 60 days. Since there were no negative effects of fly survival under the clean feeding regime, this decrease in virus titres in CFC - 1 and CFC – 2 could not be attributed to clearance of the virus from the two colonies through death of infected flies. In addition, there were no flies in the CFC - 1 and CFC - 2 colonies exhibiting GpSGHV levels normally associated with the SGH (i.e. ≥ $10^9$ viral genome copies per fly (Abd-Alla *et al.*, 2009a). While the NFC colony showed a similar trend to the standard colony, the prevalence was significantly lower than that of the main colony, with only 2.2 % (4/186) of the flies having a virus titre indicative of the presence of the SGH.

# Discussion

For successful implementation of area wide integrated pest management (AW–IPM) programme with a sterile insect technique (SIT) component in tsetse and trypanosomoses in sub-Sahara Africa, large-scale production of high quality males is

required (Hendrichs *et al.*, 2007). Unlike other insects used in SIT approaches such as fruit flies and screwworm flies, which have high reproductive rates and affordable larval diets, tsetse flies have very low productivity (Jordan and Curtis, 1968) and are strictly hematophagous (Gooding and Krafsur, 2005). This presents unique challenges for mass-production of tsetse flies for eradication of the insect and trypanosomiasis.

Tsetse colonies were initially fed on live animals (Jordan *et al.*, 1967; Mews *et al.*, 1972; Nash *et al.*, 1966), a system afterwards found incapable of *en masse* production of tsetse flies. This led to development of an *in vitro* membrane feeding system (Bauer and Wetzel, 1976; Langley, 1972a; 1972b; Langley and Maly, 1969; Mews *et al.*, 1977), which was later optimized and adopted for mass production of most tsetse fly species (Feldmann *et al.*, 1992; 1993; 1994b; Gooding *et al.*, 1997). This feeding regime greatly contributed to successful eradication of the *G. austeni* population from Unguja Island, United Republic of Tanzania (Vreysen *et al.*, 2000), since sufficient numbers of male tsetse flies could be produced. The success elicited interest in other African countries to apply SIT approach for other tsetse species found on mainland Africa, including: *G. pallidipes* (in Ethiopia) (Alemu *et al.*, 2007), *G. palpalis gambiensis* (in Senegal) (Bouyer *et al.*, 2010), *G. p. gambiensis* and *G. tachinoides* (in Burkina Faso) (Sow *et al.*, 2012), *G. austeni* and *G. brevipalpis* (in South Africa) (Vreysen, 2001). Tsetse production facilities have since been established in these and other countries, and are maintained on the membrane-feeding regime. To reduce operational costs associated with this feeding regime, up to ten rounds of fly cages are traditionally fed in sequence on the same membrane. This feeding regime is compromised due to rapid spread of GpSGHV, especially in *G. pallidipes* colonies where large amounts of infectious virus particles are released into the blood via saliva secretions (Abd-Alla *et al.*, 2010b). The rates at which GpSGHV titres and SGH prevalence increase in the colony depend on the number of feeding cycles on the same membrane. Colony handling under this feeding regime leads to mixing of pupae and newly-eclosed adults produced by younger parents with those from earlier fly generations. This eventually leads to teneral flies having varying virus titres, and often, newly-eclosed adults exhibiting fully developed SGH.

This study was carried out based on the conjecture that modifying the feeding regime could be one of the most cost - effective strategies to reduce the risk of GpSGHV infections. Data presented here clearly demonstrate that a clean feeding regime can be efficiently applied using the resources existing in tsetse fly mass rearing facilities. By changing colony management, the disadvantageous mixing of pupae and newly-eclosed adults was avoided as demonstrated by the results of the CFC - 1, CFC - 2 and NFC. For implementation of the clean feeding regime in tsetse production facilities, fly colonies could be subdivided over time into three (or more) independent (sub) colonies, with separate data recording for parents and progeny flies to monitor colony performance. The principle aim is to separate progeny of flies fed first on clean

blood from those produced by flies fed later on the same membrane. This would reduce the risk of younger generations picking up virus from the blood potentially contaminated by teneral flies with SGH fed previously.

As demonstrated by the CFC – 1 GpSGHV titres and SGH prevalence gradually decreased to undetectable levels. Consequently, flies in this colony appear to release insignificant numbers of virus particles, implying that flies in the CFC – 2 colony (fed in the second feeding round) fed on "clean blood". This sequence is repeated down to the NFC colony and eventually to the main colony (fed in the last round on the membranes). Due to the reduction in the colony size in NFC (~ 6000 flies) compared to the colony size prior to initiation of the clean feeding regime (~ 12000 flies), the number of feeds per membrane was reduced considerably, which resulted in efficient blocking in GpSGHV transmission and eventually reduction of detectable SGH.

Based on the eradication of SGH and reduction of GpSGHV titres in CFC - 1 and CFC - 2, the data presented here support the argument that vertical transmission of GpSGHV is associated with lower virus tires and morbidity (rate of SGH incidence) than horizontal virus transmission. This is interesting. It can be hypothesized that in low or moderate virus titres ($< 10^6$ virus genome copies), the reproductive system of the host fly may not be adversely affected by virus infections, meaning that the larval developmental cycle goes to completion. This vertical GpSGHV transmission, either trans-ovum or via milk gland secretions (Abd-Alla *et al.*, 2011b), maintains an asymptomatic infection status in the colonies, which, under undefined conditions, may convert to the symptomatic state, accompanied by expression of detectable SGH symptoms. SGH eventually leads to fly mortalities often associated with colony collapse. For horizontal transmission, sufficient quantities of GpSGHV have to be secreted via saliva during membrane feeding in order to be transmitted to other “healthy” flies in the colonies, and this may not necessarily lead to apparent fly mortalities.

## Acknowledgements

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# Chapter 8

# General discussion

This PhD dissertation has presented data on various aspects of the pathobiology of *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV; family *Hytrosaviridae*), a double-stranded DNA (dsDNA) virus pathogenic to tsetse flies (Diptera; Glossinidae), especially the *Glossina pallidipes* (Abd-Alla *et al.*, 2008; 2009b; 2010a). The goal of the PhD research was to investigate the infectomics of GpSGHV as an essential step towards the development of viral management strategies for application in tsetse fly mass production facilities. At the onset of the experiments described in this dissertation, it was hypothesized that the GpSGHV- and host-encoded proteins and macromolecules are expressed under conditions favourable to specific virus–host interactions to influence the viral infection process. The hypothesis was tested in a series of experiments that considerably improved the initial knowledge of GpSGHV pathobiology and has set a centre stage for future research into hytrosaviruses. This chapter provides a synopsis on the extent to which various research objectives were achieved, and discusses how some of the unresolved research questions could be tackled in future.

## Does infectomics contribute to the understanding of GpSGHV pathobiology?

Early studies had reported that GpSGHV infection as largely asymptomatic: more than 90% of asymptomatic *G. pallidipes* flies in the colonies can be PCR-positive for viral infections (Abd-Alla *et al.*, 2007a). Only 4% of the flies exhibited salivary gland hypertrophy syndrome (SGH). Yet, high prevalence of SGH was linked to colony collapses (Abd-Alla *et al.*, 2007a). It should however, be noted that the SGH prevalence in the colonies could go higher than the reported 4%. Possibly, majority of the SGH-positive flies die out from the colony before assessments of occurrence of SGH is done. In the light of these observations, the question was: how is the asymptomatic infection maintained? Further, how and under what conditions does the covert asymptomatic state convert to the overt symptomatic state? To address these questions, various matings were performed between asymptomatic (healthy or infected) and symptomatic *G. pallidipes* flies. An infection model for GpSGHV is presented in **Figure 1 A**.

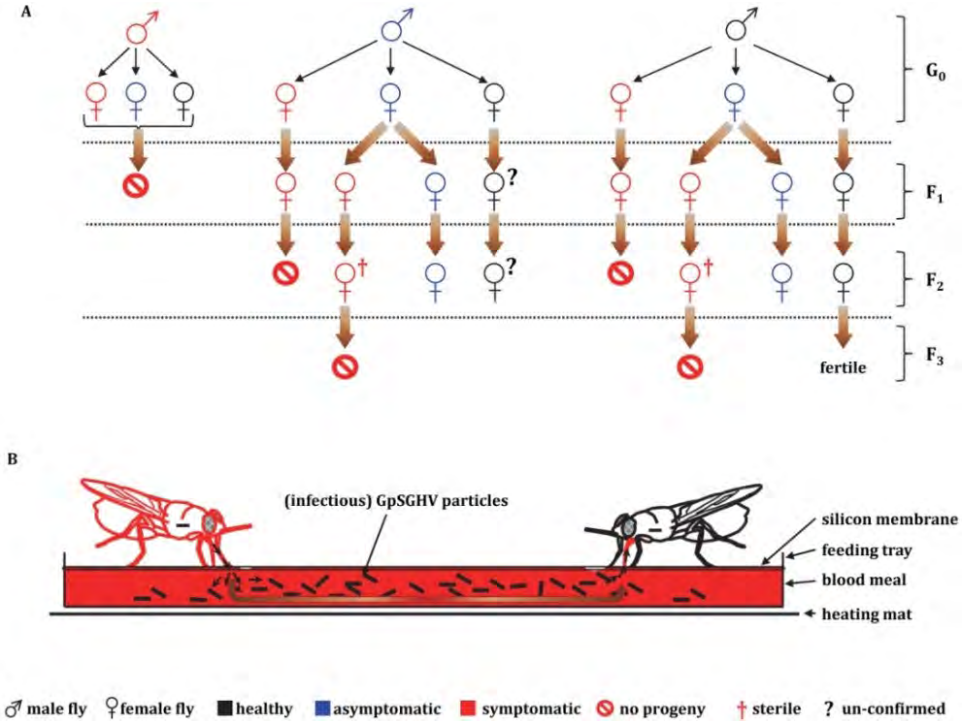


**Figure 1: A GpSGHV infection model in laboratory colonies of *G. pallidipes*.** The colony flies may either be "healthy"/non-infected – (black), asymptomatic – (blue), or symptomatic – (red). (A) Most of the symptomatic females produce few or no $F_1$ progeny (when inseminated by either symptomatic or asymptomatic males), or $F_2$ (when inseminated by healthy males). (B) During membrane feeding, GpSGHV particles released via saliva into blood meals are infectious to healthy flies. $G_0$, $F_1$, $F_2$ and $F_3$ represent parental, first, second and third progeny generations, respectively. (?) Represent progenies with unknown infection statuses (The figure is adapted from Kariithi *et al*., 2013d).

When symptomatic or asymptomatic fathers inseminate symptomatic mothers, the fly progenies die-out from the colony during the parental ($G_0$) and the $F_1$ generations, respectively. When both parents are asymptomatic, or when asymptomatic mothers are inseminated by healthy fathers, progenies die-out from the colony during the $F_2$ generation. In agreement with earlier studies (Jaenson, 1986; Sang *et al.*, 1996; 1998), GpSGHV appears to be maintained in the colonies by mother-to-progeny transmission. The lack of father-to-progeny GpSGHV transmission could be due to reduced virus transmission rates, rather than to a total failure of the transmission. This phenomenon has been demonstrated for other Diptera-infecting viruses such as *Drosophila melanogaster* virus (DMelV): failure of father-to-progeny transmission was attributed to transfer of low DMelV titers to the developing *Drosophila* embryo via the sperm (Longdon *et al.*, 2011). Although father-to-progeny GpSGHV transmission is yet to be reported, it cannot be totally ruled out.

Due to the lack of GpSGHV-free *G. pallidipes* colonies, it was not possible to predict the fate of the progeny produced by "healthy" (PCR-negative) mothers inseminated by asymptomatic fathers (marked with "?" in **Figure 1 A**). This notwithstanding, the fate of these progenies can be postulated: In 1986, Jaenson had observed that occasionally, asymptomatic *G. pallidipes* males had viable spermatozoa, which were transferable to "healthy" females. (Jaenson, 1986). Jaenson's data and the observations in **Figure 1 A** suggest that the offspring produced by the "healthy" mothers inseminated by asymptomatic fathers are most likely asymptomatic. In theory, such progenies are likely to contribute to the long-term persistence of GpSGHV infections. As demonstrated by results in chapter 6, intra-hemocoelic injection of GpSGHV into "healthy" mothers did not lead to detectable SGH symptoms in the $G_0$ generations. Despite the absence of detectable SGH symptoms, GpSGHV-injected mothers had more than $10^{11}$ viral genome copies per fly, titres way above the threshold of $10^9$ viral copies reminiscent of the occurrence of SGH symptoms (Abd-Alla *et al.*, 2009a). Taken together, these data suggest that asymptomatic individuals, especially mothers, are key players in the maintenance of GpSGHV in the tsetse fly mass production facilities.

## *Does GpSGHV 'walk' alone?*

The GpSGHV-induced pathologies; i.e. male aspermia/oligospermia (Opiyo and Okumu, 1983), under-developed female ovarioles, sterility, SGH, distorted sex ratios, reduced insemination (Jaenson, 1978a; 1978b; 1986; Opiyo and Okumu, 1983), and ultimately, collapse of *G. pallidipes* colonies (Abd-Alla *et al.*, 2007a; 2010b), raise several questions. For instance, why does GpSGHV appear to induce such serious negative impacts on colonized *G. pallidipes* compared to other tsetse fly species that are also GpSGHV-infected? Further, based on the data presented in Chapter 6, do other microorganisms such as the bacterial endosymbionts influence the outcome of GpSGHV infections? In other words, does GpSGHV 'walk ' alone on its infection path? These questions call for further extensive researches. This notwithstanding, inferences from other viral-insect systems could provide insights into the molecular mechanisms that underpin the GpSGHV infection process.

It is now well established that viruses co-infect their hosts with multiple microbes counterparts such as the maternally inherited bacterial endosymbionts, some of which are stably maintained within a host population (Dale and Moran, 2006; Duron *et al.*, 2008; Engelstadter and Hurst, 2009; Hilgenboecker *et al.*, 2008). The virus and/or symbionts co-infections of the host either can be competitive or is synergetic (Brownlie and Johnson, 2009). Often, the host's response to viral infections has been shown to be modulated by either prior or concomitant interactions with symbiont counterparts. One of the well-studied virus-insect systems is the infection of the model organism, *Drosophila melanogaster* by various viruses such as the sigma virus (DMelV) (Longdon *et al.*, 2010), and the *Drosophila* C virus (Teixeira *et al.*, 2008).

From numerous studies, it appears that DMelV and *Drosophila* engage in a co-evolutionary race, in which the host's resistance to viral infections is modulated by specific virus-host interactions (Carpenter *et al.*, 2012). In many cases, the symbionts are key players in this evolutionary race, and when the host's survival depends on maintenance of the symbionts, hosts that are devoid of these symbionts may eventually become extinct (Brownlie and Johnson, 2009).

There are now clear indications that GpSGHV infection and subsequent mother-to-progeny viral transmission may be modulated by the interplay between GpSGHV and bacterial endosymbionts (see C**hapter 6**). Notable of the results presented in chapter 6 is the apparent absence of *Wolbachia* in the laboratory stock of *G. pallidipes.* Either the laboratory stock of *G. pallidipes* is devoid of *Wolbachia*, or the titres of this endosymbiont in *G. pallidipes* are below detectable threshold. Other studies have correlated the occurrence of *Wolbachia* and GpSGHV infection in other tsetse fly spp. For instance, in *G. fuscipes fuscipes*, the levels of PCR-detectable GpSGHV titers appear to be influenced by the fly genotype, and inversely correlate with the prevalence of *Wolbachia* (Alam *et al.*, 2012). The role of *Wolbachia* during virus infection has also been investigated in other insects. For instance, high *Wolbachia* densities can provide full protection, whereas low densities confer limited protection to mosquitoes against dengue virus (DENV) infections (Lu *et al.*, 2012; Osborne *et al.*, 2009). Some economically important mosquito species such as *Anopheles* spp. are devoid of *Wolbachia* (Hughes *et al.*, 2011). Whereas none of the *Wolbachia*-infected *Aedes aegypti* tested positive for Dengue virus (DENV), 30%-100% of *Wolbachia*-free *Ae. aegypti* were infected by DENV (Hoffmann *et al.*, 2011; Moreira *et al.*, 2009). A recent study has demonstrated that, under laboratory conditions, it is possible to use *Wolbachia* to control malaria: *Wolbachia*-infected *Anopheles stephensi* were found to be refractory to the malaria parasite *Plasmodium falciparum* (Bian *et al.*, 2013). In fact, the mothers passed *Wolbachia* to the entire mosquito populations within eight generations. As a result, *Wolbachia*-infected mothers laid infected eggs, while *Wolbachia*-infected males successfully bred with infected females: if males mated with non-infected females, the resulting offspring died before hatching. The implication of this research is that in the wild, *Wolbachia*-infected mosquitoes could replace the *P. falciparum*-carrying mosquitoes, thereby successfully controlling malaria.

Why *Wolbachia* are undetectable in a laboratory stock of *G. pallidipes* (see **Chapter 6**) remains intriguing because *Wolbachia* is one of the most ubiquitous endosymbiotic bacterium occurring in insects (Jeyaprakash and Hoy. 2000). It is possible that the (potential) absence of *Wolbachia* in the *G. pallidipes* colonies explains the severe negative impact of GpSGHV, leading to development of SGH symptoms. The role of *Wolbachia* is also evident from the observation that, in the case of *Musca* domestica SGHV (MdSGHV), there is little or no *Wolbachia* (Pourali *et al.*, 2009; Kyei-Poku *et al.*, 2006). It should be noted that MdSGHV induces expression of 100% SGH symptoms in

the house fly with the flies releasing high levels of infectious viral particles between 48 and 72 h post-injection (Lietze *et al*., 2012). Further, there are no asymptomatic infections in the MdSGHV-house fly system (Geden *et al*., 2011b). In view of the shared intracellular locations of GpSGHV and tsetse microbiome (**Figure 2**) (Wang *et al.*, 2013), and the mother-to-progeny GpSGHV transmission (Boucias *et al.*, 2013b), the bacterial symbionts (perhaps except *Wolbachia*) possibly modulate GpSGHV infections in *G. pallidipes*.

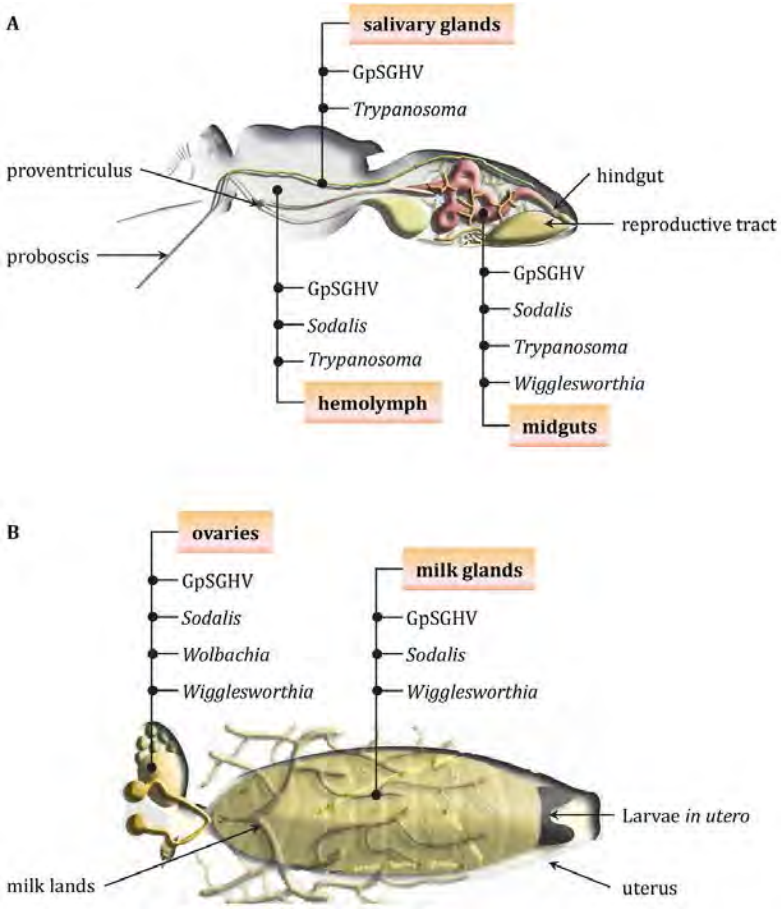


**Figure 2: Intra - and extra - cellular shared locations of GpSGHV, *Trypanosoma* and the bacterial endosymbionts in in the whole body (A) and the reproductive system of the tsetse fly (B):** *Wolbachia pipientis* resides exclusively within germ-line tissues (oocytes and embryos), while *Sodalis glossinidius* is found intra- and extracellular in diverse tissues (midguts, milk glands, and hemolymph). *Wigglesworthia glossinidia* resides intracellular in the midgut mycetome, and extracellular in milk glands. GpSGHV is widely distributed in various host tissues, but the salivary gland is the primary tissue where the virus replicates (Figure adapted from Balmand *et al.,* 2013; Kariithi *et al*., 2013d)).

One implication of the shared locations is direct competition between GpSGHV and microbiome for the same resources, or indirect disturbances by the symbiont on the cellular environment required by the virus for its replication and transmission.

## *Can symbionts contribute to creation of pathogen-refractory tsetse flies?*

In view of a potentially important role played by the bacterial symbionts in GpSGHV infection and transmission in *G. pallidipes*, the question therefore is: can the bacterial symbionts be used to create *G. pallidipes* strains that are refractory to pathogens (e.g. GpSGHV or trypanosomes)? There is sufficient evidence that manipulating the levels of symbionts can lead to a reduction in the transmission of insect-vectored diseases: For instance, *Wolbachia* can be manipulated to supress DENV transmission in *Ae. aegypti* (Hoffmann *et al.*, 2011). The *Wolbachia*-mediated increase in susceptibility of *Spodoptera exempta* (Lepidoptera; Noctuidae) to baculovirus infection has been proposed as an alternative bio-control strategy against the insect and a method of reducing pathogen susceptibility in other organisms (Graham *et al.*, 2012; Jakubowska *et al.*, 2013). It has been demonstrated that *G. m. morsitans* devoid of *Wigglesworthia* are significantly more susceptible to trypanosome infections than their wild-type counterparts (Pais *et al.*, 2008; Wang *et al.*, 2009). Recently, Weiss *et al.*, (2013) reported that the presence of microbes during intrauterine larval development is a requisite to the development of trypanosome-refractory *G. m. morsitans* phenotypes during adulthood. Potentially, the susceptibility of *Wigglesworthia*-free *G. m. morsitans* to trypanosome infections results from the fact that these flies have lesser amounts of the immune milk protein Peptidoglycan Recognition Protein (PGRP-LB) than the refractory wild-type counterparts (Wang *et al.*, 2009; Wang and Aksoy, 2012). PGRP-LB is an essential milk secretion enzyme that nourishes developing progeny, and reduction of PGRP-LB amounts (for instance by RNAi) reduces the mothers' productivity and *Wigglesworthia* densities in the milk through induction of antimicrobial peptides (Hu *et al.*, 2008; Wang *et al.*, 2009; Wang and Aksoy, 2012). These results agree with our results in transmission of GpSGHV in *G. pallidipes* (See **Chapter 6**). Manipulation of the microbiome to control pathogen transmission has been amply demonstrated in other insects. For instance in the transmission of *P. falciparum* in *Anopheles gambiae* (Boissiàre *et al.*, 2012) and *Leishmania donovani* in the sand fly *Phlebotomus argentipes* (Diptera; Psychodidae) (Hurwitz *et al.*, 2011).

Manipulating the microbiome in *G. pallidipes* might lead to a blockade or inhibition of GpSGHV replication and subsequent dissemination, making *G. pallidipes* refractory to the virus, and trypanosomes (van den Abbeele *et al.*, 2013). This presents an opportunity to develop novel technologies to control GpSGHV in tsetse fly large-scale production facilities, to the benefit of controlling trypanosomosis in sub-Saharan Africa through tsetse fly eradication programs such as the sterile insect technique (SIT).

## *Is the GpSGHV asymptomatic state a case of classical viral latency?*

The conversion of GpSGHV infection from the asymptomatic to the symptomatic states is a major factor to consider in the control of GpSGHV infections in *G. pallidipes* colonies. The question is: does the asymptomatic state represent GpSGHV latency? As an evolutionary viral strategy, viruses can utilize cues from the host and/ or environment as opportunities for dispersal and transmission. For instance, in adults of the armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae), latency provides dispersal and reproductive advantages to *S. frugiperda* nucleopolyhedrovirus (SfNPV) (Fuxa *et al.*, 1992). The logic is that as the insect host metamorphoses to adulthood, vertical transmission becomes epizootiologically more important than horizontal transmission: the virus then is transferred from one generation to another without disease symptoms (Hughes *et al.*, 1993). Latency can also be an evolutionary host strategy to control overt viral infection: the host may induce a specific RNAi response to block viral over-replication, e.g. in baculoviruses, iridoviruses and nudiviruses (Bronkhorst *et al.*, 2012; Jayachandran *et al.*, 2012; Wu *et al.*, 2011). The virus may encode miRNAs to regulate their replication or to manipulate or evade the host's immune responses (Gottwein and Cullen, 2008). During miRNA-induced latency, miRNAs keep virus-encoded protein levels to a minimum, thus facilitating evasion of the host's immune surveillance (Huang *et al.*, 2007). In some baculoviruses, latency can be stress-induced for instance by crowding, food shortage, superinfection etc. (Hughes *et al.*, 1997; Murillo *et al.*, 2011). The link between latency and 'stress' is not clearly understood and therefore, it can only be speculated upon how they are related. Possibly, in biophysical or biological stressful conditions, the host can limit protein expression as a defence strategy against virus infection. To counter this host's defence mechanism, some viruses such as *Autographa californica* multiple NPV (AcMNPV) encode a 25-kDa truncated protein kinase termed PK2, an inhibitor of eukaryotic translation initiation factor (eIF2α) kinase (Dever *et al.,* 1998). PK2 forms heterodimers with intact eIF2α kinase and as such prevents phosphorylation of eIF2α, which would otherwise lead to a blockade of protein expression (Kashles *et al.*, 1991; Ueno *et al.*, 1991). The result of this cascade of reaction is stimulation of virus replication (Dever *et al.,* 1998). In view of detection of eIF2α kinase-like proteins in the proteome of GpSGHV (See **Chapters 4**), the possibility that GpSGHV use a strategy similar to AcMNPV cannot be totally rule out. In addition to stress factors, the GpSGHV-symbiont interactions may play significant roles in establishing GpSGHV latency in tsetse flies (van den Abbeele *et al.*, 2013). Resolving the issue of GpSGHV latency and/or genome integration will provide vital clues into GpSGHV pathobiology.

## *Is the persistence of GpSGHV infections the rule rather than the exception?*

Upon infection, it would be counter-productive for a virus to initiate immediate replication and production of infectious viral progenies because this could result to killing of the host. Instead, the virus not only aims at production, but also optimal transmission, and may exploit both the horizontal and vertical transmission modes. There is a benefit for both the virus and the host to maintain a mutualistic interaction even when the biological and environmental conditions are favourable.

Murillo *et al*., (2011) reported a case where SeMNPV and MbNPV were reactivated to fully lethal forms from a covert (latent or persistent) infection state in a laboratory culture of *S. exigua*. Possibly, during the persistent infection, these viruses are actively transcribed at low level and transmitted vertically, but individual insects do not succumb to the infection. In this case, the insect culture may be reared for many years with high virus infection rates in each successful generation, but with extremely rare virus-induced mortalities and little apparent fitness cost in the host. Outbreaks of polyhedrosis may be the result of biological or physical stress as exemplified by the gypsy moth (*Lymantria dispar*) caterpillars, where regular outbreaks occur in the field due to food shortage (tree top disease caused by LdMNPV).

Latency after infection can therefore be viewed as the rule rather than the exception. In the case of GpSGHV, *Glossina* species other than *G. pallidipes* hardly show SGH symptoms in spite of the presence of high GpSGHV infections. In laboratory colonies of *G. pallidipes*, GpSGHV infections apparently disappear with proper management (see **Chapter 7**) while the virus is still there, a phenomenon that raises the obvious question: how is GpSGHV persistence infection maintained? This phenomenon could be understood if GpSGHV is able to integrate its genome into the host genome. Integration of the viral genome into that of the host has been demonstrated for other viruses, potentially as a transmission strategy (when the viruses or their genetic material enters the germ-line). For instance, Polydnaviruses are vertically transmitted as proviruses stably integrated into the genomes of the parasitic ichneumonid (Hymenoptera; Ichneumonoidae) and braconid wasps (Hymenoptera; Braconidae) (Strand, 2010). It is important to keep in mind that true latency is characterized by integration of the viral genome into host genome, with occasional production of infectious viral particles (Speck and Ganem, 2010). By integrating into the host genome, vertical viral transmission may be insured at the expense of horizontal transmission. Viral latency can also be episomal; for instance, in the case of some herpesviruses (Bennett *et al.*, 2005). On the other hand, in *D. melanogaster*, the *gypsy* elements are transmitted as 'infectious particles' from mother to progeny via oocytes (Terzian *et al.*, 2009), but without genome integration, while DENV and GpSGHV are primarily horizontally transmitted, but are also maternally transmitted (Lietze *et al.*, 2011; Rosen, 1987) without necessarily inducing disease symptoms. In the case of

baculoviruses, where viruses are also both horizontally as well as vertically transmitted the status of the virus during latency is not known (Cory and Myers, 2003).

Resolving the issue of GpSGHV latency and/or genome integration will definitely provide vital insights into the pathobiology of the virus. Potentially, if GpSGHV DNA is integrated into the maternal germ-line, then each host cell may contain a viral copy. Alternatively, the viral DNA may exist as (multiple) episomal copies in the cytoplasm of specific cells/tissues until activation.

## Ecogeography and diversity of GpSGHV

An important aspect of GpSGHV infectomics and transmission dynamics is to understand factors that generate and maintain genetic diversity of the virus under laboratory and natural environments. Early ecogeographic studies reported that the prevalence of GpSGHV infections in wild tsetse fly populations is low (0.4-15%), and depends on geographical location, seasonality, distribution and ages of tsetse species. These surveys were however, based on detection of SGH symptoms by fly dissections, which did not take into account that GpSGHV infections in the field are primarily asymptomatic (Abd-Alla *et al.*, 2009a). Possibly, in the wild tsetse populations, flies with SGH die out of the populations meaning that the actual prevalence of GpSGHV in these populations is underestimated. Furthermore, the sensitivity of the detection was not very high and could be enhanced by for instance using quantitative PCR strategies. Finally, the surveys did not investigate whether or not there is more than one GpSGHV variant circulating in the wild tsetse populations.

With advancement of molecular tools such as restriction endonucleases, targeted PCRs and deep sequencing technologies, it is possible to efficiently characterize genetic variants within and among geographic and temporal insect virus species. In chapter 3, attempts were made to address ecogeography and diversity of GpSGHV using five presumably conserved viral genes (*p74*, *pif1*, *pif2*, *pif3* and *dnapol*). On average 34% of the field-collected *G. pallidipes* samples were GpSGHV-infected, with prevalence widely ranging from 2% to 100%. Twenty-three different viral haplotypes were detected in different geographical locations. The use of the presumably conserved GpSGHV genes however, limited conclusions that could be made with regard to GpSGHV diversity, because conserved genes may exhibit limited variability. Further, multiple peaks were detected in one or more loci of the analysed genes, which were conjectured to indicate mixed GpSGHV genotypes. This conclusion is possibly inadequate because the detected multiple peaks were not identified for all the examined loci and it was not clear whether or not the peaks were repeatable, for instance, by multiple PCR amplifications.

To address the above-mentioned limitations, and to have a more complete understanding of GpSGHV genetic diversity for future research on GpSGHV evolutionary history and pathobiology, there is need to carefully select more variable GpSGHV genes, and use different tsetse fly species (e.g. *G. austeni*, *G. morsitans*, and *G. fuscipes*) sampled from a wider selection geographical locations. Proper investigations into the genetic variations of GpSGHV in field populations of tsetse flies are crucial for understanding the biology and ecology of *Hytrosaviridae*. Further investigations into the ecogeography and genetic diversity of GpSGHV would provide: (i) considerable new insights into the extent and nature of genetic variations present in GpSGHV isolates circulating in wild tsetse populations, and (ii) clues on the rates and mechanisms by which the genetic diversity occurs and is maintained. On the first point, using modern molecular epidemiological techniques, it has been possible not only to characterize genetic variants of baculoviruses in pooled samples of host larvae from different geographic insect populations, but also substantial numbers of genetic variants of baculovirus species in singly-infected individuals (Cory *et al.*, 2005). On the second point, this will be a difficult issue to address as there is currently no *in vitro* system available for genetic studies, and one has to rely on *in vivo* systems, especially in view of the fact that *Glossina* spp has a covert or latent infection of GpSGHV.

## Genomics, transcriptomics and proteomics of GpSGHV

Despite rampant GpSGHV infections, the *G. pallidipes* colony at the Joint FAO/IAEA IPCL Seibersdorf the tsetse research facility has been stable for over 20 years. Regular dissections of flies from this colony have shown a stable SGH prevalence averaging at 10% (Abd-Alla *et al.*, 2010b). This infection rate differs markedly with infection of a *G. pallidipes* colony that was established at the same facility using pupae originating from Arba Minch, Ethiopia, which collapsed in 2002. Another *G. pallidipes* colony maintained at the Tsetse Fly Rearing and Irradiation Centre, Kality, Addis Ababa, Ethiopia, revealed fluctuating (20-50%) viral infection rates (Abd-Alla *et al.*, 2010b). Taken together, these data suggest that either there is more than one GpSGHV strain circulating in these different *G. pallidipes* colonies, or the observed differences in GpSGHV pathologies may reflect differences in the environmental (insectaria conditions) and genetics of the *G. pallidipes* colony flies at Seibersdorf and Kality. It would be of interest to investigate the molecular basis underlying the pathology of the potential GpSGHV strains.

With the availability of complete GpSGHV genomes, it was important to determine which of the viral ORFs are actually expressed, and predict what roles the encoded proteins play in the virus pathobiology. This was important because the presence of protein-coding regions or ORFs in a viral genome does not necessarily imply the presence of functional proteins. For instance, frame shift mutations caused by 'indels' (insertions/deletions) in viral genomes can alter the structure and functions of the

encoded proteins, or decrease or complete loss in protein expression (Lodish *et al.*, 2000). Further, some genes may not have functional promoters. Further, the raw data produced by genomic sequences provides limited insights into the precise workings of the viral infections at the molecular and subcellular levels. Identification of proteome of the GpSGHV and the salivary secretome of GpSGHV-infected *G. pallidipes* allowed reconstruction of a dynamic view of the viral infectomics and putative virus-host interactomics (see **Chapters 4** and **5**). These data are useful in development of immune-intervention strategies against GpSGHV in *G. pallidipes* colonies (See discussion below).

## What is the story behind the replication, morphology and morphogenesis of GpSGHV?

The ultra-structural and proteomics studies presented in **Chapter 4** and **5**, and the distinct pathobiology of GpSGHV infections (See review in Lietze et al., 2011) can be used to model a (hypothetical) 'life cycle' of GpSGHV (illustrated in **Figure 3**).

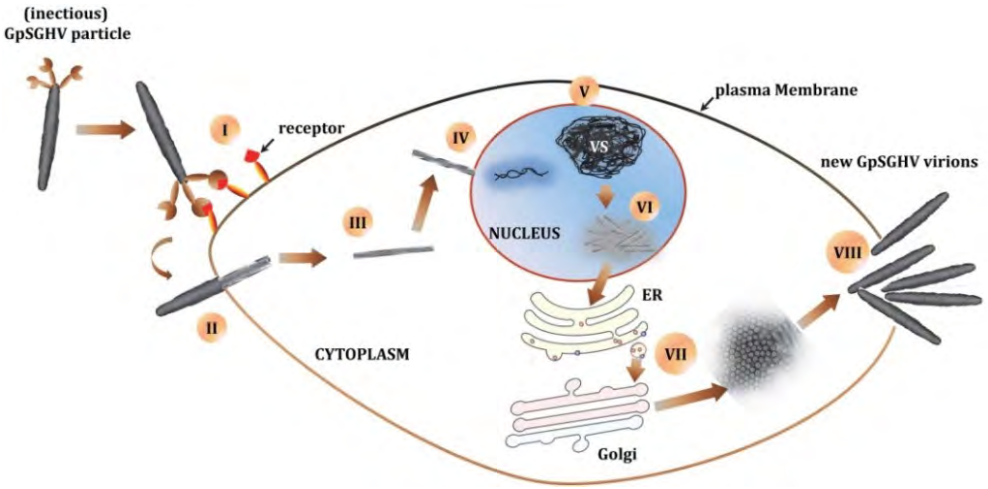


**Figure 3: Schematic representation of GpSGHV 'lifecycle':** (I) an enveloped (infectious) GpSGHV particle bind to receptors on susceptible host cell. (II) Once bound, the virus is uncoated as it enters the host cell, followed by (III) cytoplasmic trafficking of the viral nucleocapsid to the nucleus ensues, (IV) disassembly of viral nucleocapsids by partial degradation of capsid and tegument proteins and release of viral dsDNA into nucleus of host cell. (V) Once in the nucleus, the virus induces formation of virogenic stroma (VS), where viral nuclear replication occurs. (VI) After packaging of nascent viral DNA into capsids, nucleocapsids are assembled, after which they egress into the cell cytoplasm. (VII) The entire envelopment of the nascent nucleocapsids is orchestrated in the infected cell cytoplasm, possibly via the ER-Golgi system. (VIII) Egress of mature GpSGHV particles from the infected cell possibly occurs via rapture or disintegration of the plasma membranes (Figure adapted from Kariithi *et al.*, 2013d).

The exact mechanism(s) used by GpSGHV to gain entrance into the host cell are unknown. For cell entry, infectious GpSGHV particles may use *per os* infectivity factors (PIFs) (Braunagel and Summers, 2007; Peng *et al*., 2010; Slack and Arif, 2007; Slack *et al.*, 2001), or host-derived proteins on the viral envelop (Kariithi *et al.*, 2013b). Once inside the host cell, the viral envelop may be uncoated to ensure exposure of nucleocapsid proteins to the host's microtubule motor complexes for cytoplasmic traffic to the nucleus (Greber, 2005; 2009). The cytoplasmic viral traffic may be accompanied by release of tegument proteins (Kariithi *et al.*, 2013b; Luxton *et al.*, 2005). Disassembly of nucleocapsids may occur by partial degradation of the capsid and tegument proteins (Delboy *et al.*, 2008; Wolfstein *et al.*, 2006), followed by release of viral DNA into the nucleus (Rabe *et al.*, 2006). After nuclear assembly, progeny nucleocapsids translocate to the cytoplasm for envelopment, possibly via the ER-Golgi system. Mature virions egress from the infected cell via disintegration or rupture of plasma membranes: infectious virions are continuously shed into salivary gland lumens.

## Control of GpSGHV in laboratory colonies of *G. pallidipes*

To control GpSGHV, two potential strategies to manage the viral infections in *G. pallidipes* colonies were considered. The first strategy was to reduce/inhibit GpSGHV horizontal transmission by modification of the *in vitro* membrane-feeding regime used in tsetse production facilities. This strategy was successful (See **Chapter 7**). The second strategy was neutralization of GpSGHV particles secreted via saliva by infected flies during blood feeding by: (i) oral application of specific GpSGHV antibodies or oligopeptides, (ii) blocking GpSGHV attachment to the midgut receptors in the host by oral administration of antiviral drugs to inhibit GpSGHV DNA polymerase, and (iii) silencing of essential GpSGHV genes by RNAi. The principles behind selection of these strategies have been recently reviewed (Abd-Alla *et al.*, 2011b). Oral administration of the antiviral drug valacyclovir demonstrated significant reduction of viral infections in *G. pallidipes* colonies (Abd-Alla *et al.*, 2012). Application of antibodies and RNAi to mitigate GpSGHV infection need to be further optimized. Preliminary data from the immune intervention strategies to manage GpSGHV infections have been rather inconsistent; though promising, the results are not repeatable.

### *GpSGHV control: implications on SIT/AW-IMP tsetse fly control programs*

Evidently, SIT is an elegant and environmentally friendly method, and a potent birth control tactic for tsetse flies. SIT has a long and solid record of accomplishment. In its ≥ 50 years' history, SIT has helped sweep away screwworms (Lindquist *et al.*, 1992; Wyss, 2000), and various fruit flies and moth species globally (Enkerlin, 2005; Franz, 2005; Koyama *et al.*, 2004). More importantly, SIT helped wipe populations of various

tsetse species from Africa (Oladunmade *et al.*, 1990; Politzar *et al.*, 1984; Vreysen *et al.*, 2000). In all these cases, the common denominator is successful mass-production of high quality sterile male insects at reasonably low cost. In view of the successfully management of GpSGHV infections – at no additional costs in terms of equipment and staff (See **Chapter 7**) - production of sufficient numbers of sterile *G. pallidipes* males is now within reach. This presents an opportunity to revive and re-enforce campaigns with an SIT component: for instance, to eradicate *G. pallidipes* from 250,000 square kilometres of fertile land in the western and south-western Rift Valley of Ethiopia (Enserink, 2007). Some experts say that tsetse has shaped the African continent by creating the so-called "green deserts": vast, lush and fertile lands that are not in production because of tsetse fly infestation (Okhoya, 2004). Moreover, ~90% of Africa's livestock consists of herds in small villages (Otte and Chilonda, 2003). Therefore, maintaining healthy livestock can be the difference between subsistence misery and a tolerable life for the African peasant herders and their families. These facts imply that a revival of control programs against *G. pallidipes*, and other tsetse species, directly translates into availability of more animals to plough land, more nutrition, and more manure to plant crops. Simply put, the implication is poverty reduction and improved livelihoods in sub-Sahara African countries infested with trypanosome-transmitting tsetse species.

## *SIT for control of African trypanosomoses: is it a 'Pie in the Sky'?*

This PhD dissertation would be incomplete without addressing some of the pertinent concerns raised by the scientific community and the general public about application of the SIT to eradicate tsetse flies. For instance, critics have described tsetse SIT as a 'pie in the sky' project, especially considering the impoverished sub-Saharan African countries. The critics argue that SIT can cost tens of millions of dollars, and takes a long time to implement. For instance, for the highly successful eradication of *G. austeni* from the Unguja Island (Vreysen *et al.*, 2000), it took almost 10 years to build a tsetse mass-production facility and train staff, and an estimated cost of $ 5.7 million in a four- year campaign (1994-1997). Further, there are approximately 30 species and sub-species of tsetse, infesting approximately 10 million square kilometres of main land in sub-Saharan Africa (Cecchi *et al.*, 2008). If one were to extrapolate from these figures, it would take many centuries, and over $ 50 billion to eradicate just a few tsetse species. These facts paint a dark picture on SIT sustainability. However, the situation is not as bad as it is portrayed for the following reasons (1) only 8-10 of the 30 tsetse fly species are of medical and agricultural importance. (2) No one has ever claimed that SIT alone can totally eradicate tsetse flies from all over Africa; rather, when implemented in well-coordinated AW-IMP principles, SIT is highly successful. (3) There are now several tsetse production facilities in Europe and Africa, with well-trained staff that could adapt relatively easily to culturing different tsetse fly species. (4) Several countries in sub-Saharan Africa have expressed willingness to invest

resources into programs with an SIT component. Co-operation between these countries would be vital to the success of SIT: SIT is cost-effective when implemented on a large-scale, and in an organized manner. (5) If tsetse campaigns with an SIT component can help relieve African peasant farmers of their plight i.e. permanent poverty reduction, then the investments are well worth the gains. For instance, since Zanzibar was declared tsetse-free September 1997 (Vreysen *et al.*, 2000; 2011), tsetse fly and trypanosomosis appear to be out-dated. In fact, after seven years since the last tsetse was sighted in the once heavily tsetse-infested area of 1,600 square kilometres in Zanzibar, routine blood samples tested negative for trypanosomes. Consequently, milk production has tripled, local beef production has doubled, and application of animal manures has increased five-fold (Okhoya, 2004). (6) Compared to other tsetse control methods such as the use of insecticide and spray-on formulations, SIT stands out taller. SIT is environmentally benign, without toxic residues, with minimal no-target impacts, and no detectable resistance has been reported in the more than 50 years' history of large-scale SIT programmes.

## *Tsetse eradication: is it a 'ticking time-bomb' for African ecosystems?*

An equally pertinent concern is the potential ecological consequences resulting from successful tsetse eradication, e.g. loss of human and herd population immunity against African trypanosomosis. If tsetse and trypanosomosis were effectively controlled, potentially fewer people and animals would be infected, leading to decrease in population immunity and presence of more susceptible individuals. Thus, the risk of epidemics could increase, especially when control measures are lifted. Such a risk of epidemic has been reported in other viral cases. For instance, a combination of low herd immunity,' an increase in age of infection' and adoption of case-reactive vector control measures were implicated in a resurgence of DENV virus infections in Singapore (Ooi *et al.*, 2006). One could reason that removal of the target tsetse species from their natural habitat could create imbalance in the ecosystem. A counter argument is that if tsetse-dependent species do exist, SIT is species-specific, meaning that one tsetse species is eradicated from delineated areas at a time, thereby allowing time to evaluate potential ecological impacts. Further, if unacceptable consequences are observed in the future, SIT programmes are reversible before continent-wide tsetse eradication campaigns are implemented. Finally, some scientists and lobby groups argue that since tsetse fly is part of the African continent's ecosystem, its removal would bring an explosion of livestock production, and intensification of agriculture. Consequently, there would be an overall decline in biodiversity. While this may be true, it should be noted that African farmers keep large herds as security against the threat posed by african trypanosomoses. Once the threat is removed, these famers will probably shift to fewer, more productive livestock species. Besides, with proper policies and legislations natural habitats can be protected to ensure that land is used to the maximum benefits.

# Concluding remarks

From the various experiments described in this dissertation, a dynamic view of the signature features of GpSGHV pathobiology can be re-constructed (**Table 1**).

**Table 1: The footprints of GpSGHV:** The table shows the principle biological, structural and molecular characteristics of GpSGHV in comparison with MdSGHV. (Table modified from Garcia-Maruniak *et al.*, 2009).

| Main characteristics | | GpSGHV | MdSGHV | Reference(s) |
|---|---|---|---|---|
| Biological | Replication organ; replication site(s) | Salivary glands, milk glands; nucleus | Salivary glands; Nucleus | (Coler *et al.*, 1993; Geden *et al.*, 2008; Jaenson, 1978b; Jura *et al.*, 1988; Sang *et al.*, 1996) |
| | Infection phenotype | Symptomatic and asymptomatic | symptomatic | (Abd-Alla *et al.*, 2007a; Boucias *et al.*, 2013b) |
| | Symptoms besides SGH syndrome | Male and female gonadal abnormities | Under-developed ovaries | (Boucias *et al.*, 2013a; Coler *et al.*, 1993; Jura *et al.*, 1988; Lietze *et al.*, 2007) |
| | Modes of transmission | Vertical (via milk glands, trans-ovarian); horizontal (Oral/saliva) | No vertical; horizontal (Oral/saliva secretions and excreta) | (Abd-Alla *et al.*, 2007b, 2010b; Boucias *et al.*, 2013a; Coler *et al.*, 1993; Jura *et al.*, 1988; Lietze *et al.*, 2007, 2009) |
| | Sterilizing agent | Male and female infertility | Female infertility | (Lietze *et al.*, 2007; Sang *et al.*, 1999) |
| | Impact on host behaviour | Impaired feeding | Mating disruption | (Lietze *et al.*, 2007; Mutika *et al.*, 2012; Sang *et al.*, 1997) |
| | Morphogenesis | Egress by PM rapture | Egress via PM budding | (Boucias *et al.*, 2013a; 2013b; Lietze *et al.*, 2011a) |
| Structural | Virion size | 50 x 1000 nm | 65 x 550 nm | (Garcia-Maruniak *et al.*, 2008; Lietze *et al.*, 2011a) |
| | Ultra-structure | Nucleocapsid, tegument, envelop, 'spikes' | Nucleocapsid, envelop | (Kariithi *et al.*, 2013b) |
| | Virion topography | Helical surface projections | Braided, bead-like surface | (Garcia-Maruniak *et al.*, 2008; Kariithi *et al.*, 2013b) |
| Molecular | Genome size | 190, 032 bp | 124, 279 bp | (Abd-Alla *et al.*, 2008; Garcia-Maruniak *et al.*, 2008) |
| | G + C content (%) | 28 | 44 | (Abd-Alla *et al.*, 2008; Garcia-Maruniak *et al.*, 2008) |
| | No. of RFs | 160 | 108 | (Abd-Alla *et al.*, 2008; Garcia-Maruniak *et al.*, 2008) |
| | No. of shared ORFs | 41 | 37 | (Garcia-Maruniak *et al.*, 2009; Kariithi *et al.*, 2010) |
| | ORFs homologs in other large dsDNA viruses | Nudivirus (11); whispovirus (4); baculovirus (12) | Nudivirus (17); whispovirus (6); baculovirus (12) | (Abd-Alla *et al.*, 2008; Garcia-Maruniak *et al.*, 2008) |

It is clear from the data presented in this PhD dissertation that, like many other insects that serve as viral vectors, tsetse flies manage to control GpSGHV infections without completely eliminating the virus; therefore, they survive and establish a viral persistent infection. This may be advantageous for the virus as it may ensure transmission and dissemination the viral progeny. Consequently, understanding the mechanisms that underpin GpSGHV persistence in tsetse flies could help restrain the virus infections in tsetse fly mass production facilities.

Some of the envisioned strategies (i.e. immune interventions) to manage GpSGHV infections in tsetse fly colonies have so far been inconclusive. This could be attributed to several factors such as choice of the candidate protein targets to develop for instance, antibodies or oligopeptides for supplementation into blood meals. At the time these bioassays were set up, it was thought, perhaps erroneously, that the proteins encoded by the GpSGHV's ORF10 and ORF96 were major envelop proteins and therefore antisera against these proteins could immunocomplex the virus when supplemented into blood meals in such a way the that the virus would be unable to initiate infections. It is now evident, though not experimentally confirmed, that these proteins may not be on the GpSGHV envelop component. Further, it remains to be investigated the dynamics involved in the oral administration of antisera against viral proteins e.g. to determine how efficacious the approach is etc. Perhaps the apparent failures call for re-evaluation of GpSGHV management strategies. For instance, it is possible to develop viral management strategies that are not based on the components of the virus itself, but rather on host-derive molecules that interact with the virus during its 'life-cycle'. In this case, one could argue that host-derived molecules have higher chances of being efficacious because of less selection pressure, and are therefore better targets.

There is still insufficient knowledge about GpSGHV gene regulation, and the interplay of the virus, the microbiome and parasites such as trypanosomes. Nevertheless, the wealth of information availed by the researches described in this dissertation under the umbrella of many research groups (Abd-Alla and Arif, 2013), allowed successful development and application of control strategy(s) of GpSGHV in tsetse fly colonies. The data presented in this dissertation sets a centre stage for the development of more advanced strategies for tsetse fly control to eliminate trypanosomosis. The knowledge and experiences gained during the researches have been used to make recommendations on standard operational procedures on GpSGHV management in large-scale tsetse fly production facilities (See **Appendix I**; Kariithi *et al.*, 2013d). A future challenge for virologists and entomologists is to understand the natural role of GpSGHV, and the hytrosaviruses in dipteran fly ecology and evolution.

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# Appendix I

# Standard operating procedures for the management of GpSGHV in tsetse fly mass – production factories[7]

The following standard operational procedures (SOPs) are recommended on how to manage GpSGHV in large-scale tsetse fly production facilities.

1) Staff involved in tsetse rearing must be sufficiently educated to recognize SGH symptoms, and to regularly monitor variations from 'healthy' flies. Such variations include reproductive disturbances (reduced matings and egg production) and longevity (premature mortalities and prolonged larviposition cycles).

2) To establish new tsetse colonies from field-collected immature flies/pupae, progeny flies produced by SGH-free mothers should be used. Therefore, pregnant females, and the pupae produced by these mothers should be kept separately and, if possible, individually. The mothers should then be dissected to assess SGH symptoms. Where possible, the virus infection status of these mothers should be confirmed by PCR. Only fly progenies produced by SGH-free mothers should be used as seed for the new tsetse fly colonies.

3) When colonies are initiated from tsetse flies or pupae obtained from other tsetse rearing facilities, it is recommended that it should be enquired from the facility manager about the 'health' status of their colony(s), and whether the insects have been screened for SGH. If the colony has not been screened, the new insects should be quarantined and screened for viral infections before establishing them in the SGH-free colony. It is further recommended that the rearer from whom the fly or pupae were obtained should be informed on the findings of the screening efforts.

[7] The SOPs have been published as Supplementary Material in: **Kariithi, H.M.,** van Oers, M.M., Vlak, J.M., Vreysen, M.J.B., Parker, A.G., and Abd-Alla, A.M.M. **(2013).** Virology, epidemiology and pathology of *Glossina* hytrosavirus, and its control prospects in laboratory colonies of the tsetse fly, *Glossina pallidipes* (Diptera;Glossinidae). *Insects,* 4 (3): 287-319.

4) As much as possible, fly colonies should be maintained on a clean feeding regime (one feeding round per membrane). When the numbers of flies in such a colony reach the maximum number that can be handled in one feeding round, the surplus flies can be used to initiate a new colony. This second colony should be maintained on a second feeding round after feeding the first colony, and the cycle can be used to initiate other successive colonies. Importantly, the pupae produced by each of the colonies should be collected and incubated separately from the regular colonies, and the records for each of the colonies should be kept separately.

5) Excess male flies should be sampled weekly and dissected to monitor the prevalence of SGH. SGH prevalence rates of ≤ 10% are acceptable. SGH prevalence ≥ 10% should be considered as an indicator of a colony under risk.

6) For the tsetse colonies that are already established, at least 20 flies (males and females) should be sampled from each weekly unit and dissected to assess SGH prevalence. In case the SGH prevalence is ≥ 10%, blood meals offered to the colonies should be immediately be supplemented with valacyclovir. Additionally, the clean feeding regime should be implemented.

7) Since implementation of the clean feeding system does not incur additional materials and rearing staff, it is recommended to use this regime even when SGH is assumed to be eradicated from the colonies.

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# Abbreviations and definition of terms

| Abbreviation/ Term | Definitions |
|---|---|
| AAT | Animal African trypanosomiasis |
| AcMNPV | *Autographa californica* multiple nucleopolyhedrovirus |
| AlHV-1 | Alcelaphine herpesvirus-1 |
| ANOVA | Analysis of variance; a statistical analysis tool used to separate total variability found within a data set into random and systemic factors |
| ASFV | African swine fever virus |
| AW-IPM | Area-wide integrated pest management |
| CaMV | Cauliflower mosaic virus |
| DENV | Dengue virus |
| DlEPV | *Diachasmimorpha longicuadata* entomopoxvirus |
| DMelV | *Drosophila melanogaster* virus |
| dNTPs | A generic term referring to the four deoxyribonucleotides: dATP, dCTP, dGTP, and dTTP. |
| EBOV | Ebola virus |
| EBV | Epstein-Barr virus |
| EPV | Entomopoxvirus |
| ER | Endoplasmic reticulum |
| FAO | Food and Agricultural Organization of the United Nations |
| FDR | False discovery rate |
| Fecundity | Fly fertility indicated by daily pupae production per female fly |
| GpSGHV | *Glossina pallidipes* salivary gland hypertrophy virus |
| Haplotype | A group of viral isolates possessing an identical set of alleles at separate loci in the gene sequence |
| HAT | Human African trypanosomiasis |
| HCMV | Herpes human cytomegalovirus |
| HCV | Acute hepatitis C virus |
| HIV-1 | Human immunodeficiency virus 1 |
| HSD Test | Tukey's Honestly Significant Difference (HSD) Test; A conservative pairwise comparison technique to analyse patterns of differences between means of statistical data sets. |
| HSV-1 | Herpes simplex virus-1 |
| *Hytrosaviridae* | A virus family name derived from "*Hy*per*tro*phia *s*ialo*a*denitis", a Greek word for "salivary gland inflammation". |
| IAEA | International Atomic Energy Agency |
| iBAQ | intensity-Based Absolute Quantification |

| Abbreviation/ Term | Definitions |
|---|---|
| Infectome | A generic term that describes the genome-scale data emerging from high-throughput technologies, e.g. genomics, proteomics, interactomics |
| Infectomics | Generic term describing dynamic expression profile changes in virus and its host during infections |
| IPCL | Insect Pest Control Laboratories |
| KARI | Kenya Agricultural Research Institute |
| KETRI | Kenya Trypanosomiasis Research Institute |
| KSHV | Kaposi's sarcoma-associated herpesvirus |
| Latency | A viral infection that does not produce visible disease symptoms, but the virus may be transmitted. |
| LbFV | *Leptopilina boulardi* filamentous virus |
| LC-MS/MS | Liquid chromatography coupled to electrospray and tandem mass spectrometry |
| LCMV | Lymphocytic choriomeningitis virus |
| MAFFT | A method for **m**ultiple sequence **a**lignment based on the **f**ast **F**ourier transform |
| MARV | Marbugvirus |
| MCMV | Murine cytomegalovirus |
| MdSGHV | *Musca domestica* salivary gland hypertrophy virus |
| MeSGHV | *Merodon equestris* salivary gland hypertrophy virus |
| MeV | Measles virus |
| MIMI | *Acanthamoeba polyphaga* mimivirus (mimivirus) |
| MNV-1 | Murine norovirus-1 |
| Mo-MuLV | Moloney murine leukaemia virus |
| M-PMV | Mason-Pfizer monkey virus |
| NLS-BP | Bipartite nuclear localization signal |
| ODV | Occlusion derived virus |
| PAMPs | Pathogen-associated molecular patterns |
| PAUP | Phylogenetic analysis using parsimony; *in silico* phylogenetics for inferring evolutionary trees |
| PDVs | Polydnaviruses |
| PGRP-LB | Peptidoglycan recognition protein LB |
| PIF | *per os* infectivity factor |
| PLRV | Potato leaf roll virus |
| PMSF | Phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride |
| proPO-AS | Prophenol-oxidase-activating system |
| qPCR | Quantitative polymerase chain reaction |
| RNAi | RNA interference; a sequence-specific process in which the introduction of a dsRNA into a cell inhibits expression of a target gene. |
| RSV | Rous sarcoma virus |
| RV | Rabies virus |

| Abbreviation/ Term | Definitions |
|---|---|
| SaPIs | *Staphylococcus* aureus pathogenecity islands |
| SARSCoV | Severe acute respiratory syndrome coronavirus |
| Scotophase | Artificially-induced dark phase in a light-darkness cycle |
| SeV | Sendai virus |
| Sf6 | *Shigella flexneri* 6 |
| SfNPV | *Spodoptera frugiperda* nucleopolyhedrovirus |
| SGH | Salivary gland hypertrophy |
| SGHV | Salivary gland hypertrophy virus |
| SIT | Sterile insect technique |
| SIV | Simian immunodeficiency virus |
| SNPs | Single nucleotides polymorphisms |
| SV-40 | Simian virus 40 |
| TEM | Transmission electron microscopy |
| Teneral fly | Newly-eclosed, immature fly (after exclusion from pupa) |
| TMEV | Theiler's murine encephalomyelitis virus |
| TnNPV | *Trichoplusia ni* nuclear polyhedrovirus |
| TYLCV | Tomato yellow leaf curl virus |
| VEE | Venezuelan equine encephalitis |
| Virogenic stroma | A virus-induced chromatin-like network of electron-dense filaments within an infected cell nucleus |
| Vitellogenesis | Process of egg formation via deposition of nutrients in female oocytes |
| VLPs | Virus-like particles; Non-infectious biological constructs that are self-assembled from virus's coat proteins; mostly used as vaccine's component. |
| VV | Vaccinia virus |
| WHO | World Health Organization |
| WNV | West Nile virus |
| WSSV | White spot syndrome virus |

# Summary

The salivary gland hypertrophy syndrome (SGH) is a major scourge of laboratory colonies of the tsetse fly *Glossina pallidipes* (Diptera; Glossinidae). SGH is caused by the *G. pallidipes* salivary gland hypertrophy virus (GpSGHV; family *Hytrosaviridae*). Currently, the family *Hytrosaviridae* consists of only one other family member, a similar virus of the housefly *Musca domestica* (Diptera; Muscidae) (MdSGHV). However, detection of SGH in the plant-feeding bulb fly *Merodon equestris* (Diptera; Syrphidae) and in the parasitic wasp *Diachasmimorpha longicuadata* (Hymenoptera; Braconidae) implies that the *Hytrosaviridae* may contain other family members. The current shortage of field-based insect virologists, combined with the SGHVs' intrinsic properties (chronic covert infection of adult stages) may have hindered discoveries of other hytrosaviruses.

This PhD study has traced the history of SGHVs, and investigated the structural and functional genomics and proteomics (infectomics) of GpSGHV. A series of experiments was performed to: (i) determine GpSGHV epidemiology and genetic diversity; (ii) identify which of the putative GpSGHV genes are truly expressed into proteins; (iii) determine to what extent the virally-encoded, and host-derived proteins contribute to GpSGHV ultrastructure and pathobiology; (iv) investigate the tripartite interactions of GpSGHV, microbiome and the host and how these interactions influence the GpSGHV infection process; and (v) to develop a protocol to prevent the transmission of GpSGHV in (large scale) laboratory cultures.

The prevalence of GpSGHV infections in the natural tsetse populations is low (0.4-15.6%), and varies with the geographical location, seasonality, distribution, age and species of the tsetse flies. In wild *G. pallidipes* populations: GpSGHV diversity is low and the virus distribution is somewhat confused i.e. some GpSGHV haplotypes only occur in certain geographical locations. On the other hand, in laboratory colonies of *G. pallidipes* GpSGHV infection prevalence can be as high as 100%. In the fly colonies, all male flies exhibiting SGH are almost fully sterile. Female flies with SGH produce offspring: most (if not all) of the progeny flies produced by the SGH-positive females are also SGH-positive. Asymptomatic females produce a small proportion of SGH-positive progeny flies: if the progeny are females, the next generation ($F_2$) is sterile.

GpSGHV infection can be either asymptomatic or symptomatic. While the former does not have any apparent fitness cost to the host, the latter causes reproductive dysfunction in male and female *G. pallidipes* flies. Under laboratory conditions horizontal (fly-to-fly) GpSGHV transmission is promoted by the feeding regime using

membranes that is routinely used in tsetse mass production facilities. In the wild populations of *G. pallidipes*, GpSGHV is maintained most likely by the vertical (mother-to-progeny) transmission, of which asymptomatic mothers play significant roles in the viral persistence within the insect populations. Similar to other insect viruses, GpSGHV infects its host concurrently with bacterial symbionts (*Wigglesworthia*, *Sodalis*, and/or *Wolbachia*). The symbionts might play important roles in the infection and trans-generational transmission of the virus. For instance, antibiotic-mediated removal of the tsetse fly symbionts blocks mother-to-progeny GpSGHV transmission. Potentially, there may be a role of the microbial communities in the 'switch' from asymptomatic to symptomatic GpSGHV infection. Whether GpSGHV asymptomatic infection represents viral latency is currently being investigated.

A mature GpSGHV particle constitutes of four morphologically distinct structures: (i) an inner nucleocapsid core enclosing the double-stranded viral DNA genome; (ii) a fragile outer (glyco-) protein envelop; (iii) an electron-dense proteinaceous tegument that separates the nucleocapsid from the envelope; (iv) an outer helical sub-structure with proteinaceous projections. The GpSGHV genome encodes at least sixty-one proteins, forty-five of which constitute virion proteins. Further, the purified GpSGHV virion contains numerous host-derived (cellular) proteins, some of which appear to be specifically incorporated into the virus particles, reflecting their potential roles in the viral morphogenesis.

The successful implementation of GpSGHV management protocols in laboratory colonies of *G. pallidipes* has profound implications for tsetse and trypanosomiasis eradication in sub-Sahara Africa. For instance, an opportunity is now available to produce the large numbers of sterile males required for the sterile insect technique (SIT) programs to eradicate *G. pallidipes* (an important vector of African trypanosomiasis) from the lush and fertile Ethiopian Rift Valley lands. Finally, the research described in this thesis opens new frontiers of research on hytrosaviruses, such as the interactions of these viruses with their hosts and the microbiome.

# Samenvatting

Zwelling van speekselklieren (salivary gland hypertrophy syndrome = SGH) van de tseetseevlieg *Glossina pallidipes* (Diptera: Glossinidae) is een belangrijk probleem bij laboratoriumkweken van deze vliegen. De ziekte SGH wordt veroorzaakt door een virus: het salivary gland hypertrophy virus (GpSGHV) (familie *Hytrosaviridae*). Op dit moment omvat de familie *Hytrosaviridae* nog een ander familielid, een soortgelijk virus van de huisvlieg *Musca domestica* (Diptera: Muscidae) (MdSGHV). Echter, het optreden van SGH-symptomen bij de zich op planten voedende uienvlieg *Merodon equestris* (Diptera: Syrphidae) en de parasitaire wesp *Diachasmimorpha longicuadata* (Hymenoptera; Braconidae) doet vermoeden dat de familie Hytrosaviridae wellicht nog meer leden kent. De beperkte veldkennis van insectenvirologen, gecombineerd met het feit dat twee van de intrinsieke eigenschappen van SGHV's (chronische infectie van volwassen vliegen en afwezigheid van duidelijke ziektesymptomen), staat het ontdekken van nieuwe hytrosavirussen in de weg.

In dit proefschrift werd de geschiedenis van SGHV's nagegaan en werd het infectiegebeuren (infectomics) rondom GpSGHV onderzocht (structurele en functionele genomica, proteomica). Een serie experimenten werd uitgevoerd om: (i) de epidemiologie en genetische diversiteit van GpSGHV te onderzoeken; (ii) te bepalen welke van de hypothetische open leesramen in de DNA-basenvolgorde van het virus feitelijk in eiwit worden vertaald; (iii) na te gaan in welke mate de virus-gecodeerde en gastheereiwitten bijdragen aan de (ultra)structuur en de pathobiologie van het virus; (iv) te onderzoeken in welke mate microbiota bijdragen aan de interacties tussen virus en gastheer, en hoe deze interacties het infectieproces beïnvloeden; en (v) een protocol te ontwikkelen om virusoverdracht in laboratoriumkweken te voorkomen.

Het vóórkomen van GpSGHV-infecties in natuurlijke tseetseevliegpopulaties is relatief laag (0,4-2%) en varieert afhankelijk van de plek (geografische positie), het seizoen, de populatieverdeling, de leeftijd en het soort tseetseevliegen. In wilde *G. pallidipes*-populaties is de diversiteit tussen GpSGHV-isolaten gering en is de verdeling van de genotypen niet eenduidig d.w.z. sommige GpSGHV-haplotypen komen in bepaalde gebieden voor, maar weer niet in andere streken. Anderzijds kan in laboratoriumkweken van *G. pallidipes* GpSGHV wel in alle dieren vóórkomen. In tseetseevliegkweken zijn alle mannetjes met SGH vrijwel helemaal steriel. Vrouwtjesvliegen met SGH produceren nog steeds nakomelingen: de meeste, zo niet alle nakomelingen van vrouwtjes met SGH krijgen ook SGH. Vrouwtjes zonder SGH-

symptomen produceren maar een kleine hoeveelheid SGH-positieve nakomelingen: als deze nakomelingen vrouwtje zijn, dan is de volgende generatie ($F_2$) steriel.

GpSGHV-infecties kunnen dus symptomatisch (SGH) of asymptomatisch zijn. Terwijl de eerste categorie ogenschijnlijk geen 'fitness'-kosten met zich meebrengt voor de vlieg, kent de laatste categorie van de tseetseevliegen een niet goed functionerend reproductievermogen bij zowel mannetjes als vrouwtjes. Onder laboratoriumomstandigheden wordt de horizontale overdracht van GpSGHV (van vlieg op vlieg) bevorderd door te voeden via membranen, een techniek ('membrane feeding') die routinematig wordt toegepast bij massakweken van tseetseevliegen. Bij veldpopulaties van de tseetseevlieg wordt GpSGHV waarschijnlijk gehandhaafd via verticale virusoverdracht (van moeder op kind), waarbij de asymptomatische vrouwtjes een belangrijke rol spelen bij de handhaving van het virus in de tseetseevliegpopulaties. Net zoals bij andere insectenvirussen het geval is, heeft GpSGHV bij infectie te maken met aanwezige bacteriële endosymbionten (*Wigglesworthia*, *Sodalis*, en/of *Wolbachia*) in de gastheer. Deze endosymbionten spelen misschien een belangrijke rol bij de virusinfectie en de overdracht ervan op volgende tseetseevlieggeneraties. Eliminatie van deze microbiota via behandeling, met antibiotica bijvoorbeeld verhindert de overdracht van het virus van de moeder naar haar nakomelingen. Wellicht is er ook een rol van deze microbiota in de 'switch' van een asymptomatische naar een symptomatische virusinfectie. In hoeverre een asymptomatische GpSGHV-infectie een latente infectie vertegenwoordigt moet nader onderzoek duidelijk maken.

Het complete virusdeeltje bestaat uit een viertal morfologisch herkenbare structuren: i) een intern nucleocapside, waarin het dubbelstrengig DNA van het virus is opgenomen; (ii) een kwetsbare buitenmembraan (envelop), die virus-gecodeerde (glyco)proteinen bevat; (iii) een elektronendichte eiwitachtige tussenstructuur (tegument), dat het nucleocapside scheidt van de envelop; en (iv) een helixachtige substructuur aan de buitenkant met eiwitachtige uitsteeksels. Het genoom van GpSGHV codeert voor tenminste eenenzestig eiwitten, waarvan er vijfenveertig onderdeel zijn van het virion. Daarnaast bevat het gezuiverde GpSGHV-virion een groot aantal gastheereiwitten, waarvan er enkele wellicht specifiek in het GpSGHV-virusdeeltje zijn opgenomen en een reflectie vormen van de weg die het virusdeeltje in de cel heeft afgelegd bij zijn ontstaan.

De succesvolle implementatie van GpSGHV-managementprotocollen bij laboratoriumkweken van tseetseevliegen heeft grote betekenis voor het uitroeien van tseetseevliegen en slaapziekte in sub-Sahara Afrika. Het wordt nu bijvoorbeeld mogelijk om grote aantallen steriele tseetseevliegmannetjes te produceren ten behoeve van 'sterile insect technique (SIT)' ter bestrijding van *G. pallidipes*, een belangrijke vector van de slaapziekte in Afrika, in de weelderige en vruchtbare

Riftvallei in Ethiopië. Ten slotte, het onderzoek beschreven in dit proefschrift opent nieuwe uitdagingen voor onderzoek aan hytrosavirussen, zoals de interactie van deze virussen met hun gastheren en het daarin aanwezige palet van micro-organismen.

# Muhtasari

Uvimbe wa tezi la mate (SGH) ni janga kubwa kwa ufugaji wa mbung'o katika maabara, hasa kwa kabila la mbun'go waitwao *Glossina pallidipes* (Diptera; Glossinidae). SGH inasababishwa na virusi vinajulikana kama '*G. pallidipes* salivary gland hypertrophy virus' (GpSGHV; vilivyo katika familia/koo za *Hytrosaviridae*). Hivi sasa, katika ukoo wa *Hytrosaviridae*, kuna mwanachama mwingine mmoja tu ambaye ni virusi vinajulikana kama *Musca domestica* SGHV (MdSGHV), anaoambukiza inzi wa nyumbani, *Musca domestica* (Diptera; Glossinidae). Hata hivyo, kuonekana kwa dalili za SGH katika nzi anayekula mimea, *Merodon equestris* (Diptera; Syrphidae), na katika vimelea vya wadudu aina ya nyigu, *Diachasmimorpha longicuadata* (Hymenoptera; Braconidae) ina maana kwamba ukoo wa *Hytrosaviridae* unaundwa na kundi la wanachama wengi zaidi. Uhaba wa sasa wa watafiti wa virusi katika wadudu, pamoja na usiri katika maambukizi ya SGHVs (maambukizi sugu katika wadudu waliokomaa [wazima], na kukosekana kwa dalili dhahiri za SGH) ndicho kimekuwa kikwazo katika kutambua kabila zingine ambazo zinajumuika ndani ya ukoo wa *Hytrosaviridae*.

Utafiti huu wa shahada ya uzamivu (PhD) umefuatilia kwa undani historia ya SGH, hasa kuchunguza kazi na miundo ya vinasaba na protini za virusi vya GpSGHV. Katika utafiti huu wa shahada ya uzamivu, majaribio kadhaa yalifanyika katika utafiti huu ili: (i) Kuchunguza namna virusi vya GpSGHV vinavyooenea, na vinasaba tofauti vya GpSGHV; (ii) Kubaini vinasaba vya GpSGHV ambavyo hutafsirika katika taswira halisi ya protini; (iii) Kuchunguza jinsi protini za GpSGHV, pamoja na protini za mbung'o huchangia muundo na biolojia ya GpSGHV; (Iv) Kuchunguza mwingiliano ya GpSGHV / bakteria / mbung'o, na jinsi haya maingiliano yanavyohusika katika mchakato wa maambukizi ya GpSGHV.

Kiwango cha vimelea sababishi vya maambukizi ya magonjwa yanayosababishwa na virusi vya GpSGHV katika mbung'o walioko porini ni ya kiwango cha chini (0.4-2%). Aidha, kiwango hiki hutofautiana kulingana na maeneo ya kijiografia, misimu, umri wa mbung'o, pamoja na kabila za mbung'o. Katika mbung'o pori kabila la *G. pallidipes,* utofauti wa vinasaba vya GpSGHV ni wa kiwango cha chini sana, na usambazaji wa virusi hivi ni wa kuchanganyikiwa kiasi ya kwamba, baadhi ya aina ya virusi vya GpSGHV kutokea katika maeneo fulani kijiografia na si kwa maeneo mengine. Kwa upande mwingine, Katika mbung'o wafuguao maabara wa kabila hili la *G. pallidipes*, kiwango cha maambukizi inaweza kufikia asilimia mia moja. Na katika mbung'o hawa wafuguao, karibu mbung'o dume wote wanaoonyesha dalili za SGH huwa ni tasa. Kwa upande mwingine, watoto wengi (kama si wote) wanaozaliwa na majike ya mbung'o wenye dalili dhahiri za SGH, huwa na maambukizi dhahiri ya SGH. Sehemu ndogo ya

kizazi cha kwanza ($F_1$) cha majike ya mbung'o ambao wana ugonjwa usiodhahiri kwa macho, bado huwa na maambukizi ya SGH; na kama kizazi hiki cha kwanza ni majike, ndiposa kizazi hiki cha pili ($F_2$) kinakuwa tasa bila uwezo wa kuzalisha au kuzaa.

Maambukizi ya GpSGHV yanaweza kuwa yaliyofichika bila kuonyesha dalili yeyote, ama huonyesha dhahiri dalili za SGH. Ingawaje maambukizi yasiyo na dalili dhahiri za SGH hayana gharama yoyote kwa tija na hali ya maisha ya mbung'o, maambukizi ambayo yana dalili dhahiri za SGH huleta athari kubwa katika uzazi wa madume na majike ya mbung'o wa kabila la *G. pallidipes*. Maambukizi ya GpSGHV kutoka kwa mbung'o mmoja hadi mwingine huongezeka na kuharakishwa na mfumo wa ulishaji wa mbung'o kutumia damu iliyomwagwa katika masinia maalumu ambayo hutumiwa kwa kawaida katika maabara kubwa za uzalishaji wa mbung'o. Kwa mbung'o pori wa *G. pallidipes*, maambukizi ya virusi vya GpSGHV husababishwa na maambukizi ya kutoka kwa mama hadi kwa kizazi chake, ambapo mbung'o jike mwenye dalili dhahiri za SGH huhusika sana katika kusababisha usugu wa virusi. Sawa na virusi wengine wanaoambukiza wadudu, GpSGHV huambukiza mbung'o kwa ushirikiana na viumbe wengine wadogo kama vile bakteria (*Wigglesworthia*, *Sodalis*, na / au *Wolbachia*). Utafiti huu umebainisha kwamba bakteria hawa huchangia majukumu muhimu katika maambukizi ya virusi kutoka kwa kizazi hadi jizazi cha wadudu. Kwa mfano, kuondolewa kwa bakteria hawa kutoka kwa mbung'o kwa kutumia antibiotiki huzuia maambukizi ya virusi vya GpSGHV kutoka kwa mbung'o jike kwenda kizazi chake. Uchunguzi huu unaonyesha majukumu ya jamii za bakteria katika 'kubadili' kutoka dalili za SGH isiyodhahiri kwa ile dalili dhahiri. Uchunguzi unaendelea kwa sasa kubainisha kama kweli maambukizi ya SGH yasiyo na dhariri yanawakilisha maambukizi yasiyochangamfu ya GpSGHV, au yanawakilisha maambukizi ya ngazi ya chini sana tu ya kuambukizwa na hivi virusi.

Virusi vya GpSGHV ambavyo vimekomaa huwa na vipengele vinne tofauti katika muundo wao: (I) Kipengele cha muundo msingi kinachoitwa 'nucleocapsid', na hunasa vinasaba maalumu ya virusi; (ii) Kipengele muundo cha nje kabisa kinajumuishwa na misokoto midogo ya makadirio ya protini; (iii) Chini ya safu ya kipengele hiki cha nje kuna utando maridadi unaojumuisha protini; (iv) Kipengele cha muundo amofasi kinachojumuisha protini nyingi (kinachoitw 'tegument') hutenganisha muundo msingi ndani kutoka utando wa nje wa GpSGHV. Vinasaba ya virusi vya GpSGHV hutoa protini angalau sitini na moja, arobaini na tano ambazo hupatikana katika muundo wa virusi. Zaidi ya hayo, virusi vilivyosafishwa vya GpSGHV vina protini nyingi zinazokusanywa na virusi hivi kutoka seli za wadudu kama mbung'o. Matokeo ya utafiti ya protini zilizokusanywa na virusi vya GpSGHV umeonyesha kuwa baadhi ya protini hizi huingizwa hasa katika chembe ya virusi GpSGHV iliyokomaa na kwa hiyo, hizi protini zinakuwa na majukumu muhimu wakati wa mabadiliko ya ukuaji/umofojenesi ('morphogenesis') wa virusi.

Mafanikio ya utekelezaji wa itifaki ya kudhibiti maambukizi ya GpSGHV katika mbung'o wa kabila ya *G. pallidipes* walioko maabara inaleta matumaini makubwa ya kutokomeza mbung'o na malale katika Africa iliyo kusini mwa jangwa la Sahara. Kwa mfano, mafanikio hii inatoa fursa ya kuzalisha idadi kubwa ya madume ya mbung'o ambayo hayawezi kuzalisha watoto baada ya kupanda majike ya mbung'o yasiyopandwa bado yaliyoko porini. Njia hii inaweza kutumika kutokomeza mbung'o wa kabila la *G. pallidipes* kutoka ardhi ya kilimo yenye rutuba ya Bonde la Ufa la Afrika ilyomo kusini-magharibi nchini Ethiopia. Hatimaye, utafiti ulioelezwa katika utafiti huu wa shahada ya uzamivu umefungua mipaka mipya katika tafiti zaidi za SGHVs, na jinsi virusi hivi vinavyotegemea uwepo wa viumbe wengine wakati wa maambukizi.

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# List of publications

**Publications in Peer-reviewed Journals**

1. **Kariithi, H.M**.; van Oers, M.M., Vlak, J.M., Vreysen M.J.B, Parker A.G., and Abd-Alla, M.M.A. (**2013**). Virology, epidemiology and pathology of *Glossina* hytrosavirus, and its control prospects in laboratory colonies of the tsetse fly *Glossina pallidipes* (Diptera: Glossinidae). *Insects,* 4 (3): 287-319

2. Seth, B.C.; Binepal, Y.S.; Ruder, M.G.; **Kariithi, H.M**.; Linthicum, K.J.; Ayamba, A.; Small, J.; Tucker, C.J.; Ateya, L.; Oriko, A.A.; Gacheru, S., and Wilson, W.C. (**2013**). Rift Valley fever risk map model and sero-prevalence in selected wild ungulates and camels from Kenya. *PLoS ONE*, 8 (6): e66626.

3. Abd-Alla, M.M.A.; **Kariithi, H.M**.; Mohamed, A.H.; Lapiz, E.; Parker, A.G., and Vreysen, M.J.B., (**2013**). Managing hytrosavirus infections in *Glossina pallidipes* colonies: Feeding regime affects the prevalence of the salivary gland hypertrophy syndrome. *PLoS ONE*, 8 (5): e61875.

4. Boucias, D.G.; **Kariithi, H.M**.; Bourtzis, K.; Schneider, D.I.; Kelly, K.; Miller, W.J.; Parker, A.G., and Abd-Alla, M.M.A. (**2013**). Trans-generational transmission of the *Glossina pallidipes* hytrosavirus depends on the presence of a functional symbiome. *PLoS ONE*, 8 (4): e61150.

5. **Kariithi, H.M**.; van Lent, J.W.; Boeren, S.; Abd-Alla M.M.A.; İnce, A.İ; van Oers M.M., and Vlak, J.M. (**2013**). Correlation between structure, protein composition, morphogenesis and cytopathology of *Glossina pallidipes* salivary gland hypertrophy virus*. J. Gen. Virol.,* 94 (1): 193-208.

6. **Kariithi, H.M**.; Ahmadi, M.; Parker, A.G.; Franz, G.; Ros, V.I.D.; Haq, I.; Elashry, A.M.; Vlak, J.M.; Bergoin, M.; Vreysen, M.J.B., and Abd-Alla, M.M.A. (**2012**). Prevalence and genetic variation of salivary gland hypertrophy virus in wild populations of the tsetse fly *Glossina pallidipes* from southern and eastern Africa. *J. Invertebr. Pathol.*, 112 (Suppl. 1): S123-S132.

7. **Kariithi, H.M**.; İnce, A.İ.; Abd-Alla, M.M.A; van Oers, M.M; Vlak, J.M., and Bergoin, M. (**2012**). Proteomic footprints of a member of Glossinavirus (*Hytrosaviridae*): An expeditious approach to virus control strategies in tsetse factories. *J. Invertebr. Pathol.*, 112 (Suppl. 1): S26-S31.

8. **Kariithi, H.M**.; İnce, A.İ.; Boeren, S.; Abd-Alla, M.M.A.; Parker, A.G.; Aksoy, S.; Vlak, J.M., and van Oers, M.M. (**2011**). The salivary secretome of the tsetse fly *Glossina pallidipes* (Diptera: Glossinidae) infected by salivary gland hypertrophy virus. *PLoS Negl. Trop. Dis.*, 5 (11): e1371.

9. **Kariithi, H.M**.; İnce, A.İ.; Boeren, S.; Vervoort, J.J.M.; Bergoin, M.; van Oers, M.M.; Abd-Alla, M.M.A., and Vlak, J.M. (**2010**). Proteomic analysis of *Glossina pallidipes* salivary gland hypertrophy virus virions for immune intervention in tsetse fly colonies. *J. Gen. Virol.*, 91 (12): 3065-3074.

10. Abd-Alla, M.M.A,; **Kariithi, H.M**.; Parker, A.G.; Robinson, A.S.; Kiflom, M.; Bergoin, M., and Vreysen, M.J.B. (**2010**). Dynamics of the salivary gland hypertrophy virus in laboratory colonies of *Glossina pallidipes* (Diptera: Glossinidae). *Virus Res.*, 150 (1-2): 103-110.

11. **Kariithi, H.M**.; Binepal, Y.S.; Wilson, W.C.; Soi, R.K.; Ateya, L.O., and Oriko, A.A. (**2010**). Sero-surveillance of Rift Valley fever in sheep and goat flocks in high risk areas in Kenya. *Kenya Veterinarian*, 34: 13-19.

**Conference Proceedings**

1. Boucias, D.G.; **Kariithi, H.M**.; Bourtzis, K.; Schneider, D.I.; Kelly, K.; Miller, W.J.; Parker, A.G., and Abd-Alla, M.M.A. (**2013**). Trans-generational transmission of the *Glossina* hytrosavirus depends on the presence of a functional symbiome. *In: Abstract Book of the EFS-EMBO Symposium; Integrated insect immunology: From basic biology to environmental applications*, Polonia Castle in Pultusk, Poland, September 23-28, **2013**, p. 62.

2. **Kariithi, H.M.;** van Lent, J.W.; Boeren, S.; Abd-Alla, M.M.A.; İnce, A.İ.; van Oers, M.M., and Vlak, J.M. Structure, protein composition, morphogenesis and cytopathology of Glossina pallidipes hytrosavirus. *In: Abstract Book of the 45th Annual Meeting of the Society for Invertebrate Pathology*, Buenos Aires, Argentina, August 5 -9, **2012**, p. 97**.**

3. Abd-Alla, A.M.M.; **Kariithi, H.M**.; Parker, A.G.; Bonning, B.C.; Vlak, J.M.; Bergoin, M., and Vreysen, M.J.B. Potential management strategies to suppress salivary gland hypertrophy virus (SGHV) infection in *Glossina pallidipes* tsetse flies rearing. *In: Abstract Book of the 44th Annual Meeting of the Society for Invertebrate Pathology*, Halifax, Nova Scotia, Canada, 7 - 11 August, **2011**, p. 46.

4. **Kariithi, H.M**.; İnce, A.İ.; Boeren, S.; Abd-Alla, M.M.A.; van Oers, M.M.; Aksoy, S., and Vlak, J.M. The salivary secretome of salivary gland hypertrophy virus-infected tsetse fly *Glossina pallidipes* (Diptera: Glossinidae) *In: Abstract Book of the 44th Annual Meeting of the Society for Invertebrate Pathology*, Halifax, Nova Scotia, Canada, 7 - 11 August, **2011**, p. 45.

5. İnce, A.İ.; **Kariithi, H.M.;** Boeren, S.; Vervoort, J.J.M.; Bergoin, M.; Vlak, J.M.; Abd-Alla, M.M.A., and van Oers, M.M.. The proteome of *Glossina pallidipes* salivary gland hypertrophy virus (Hytrosaviridae) virions. *In: Proceedings of the 43rd Annual Meeting of the Society for Invertebrate pathology*, Trabzon, Turkey, 11 - 15 July, **2010**, p. 36.

# PE&RC education certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities).

**Review of literature (6 ECTS)**

- Proteomic footprints of a member of *Glossinavirus* (*Hytrosaviridae*): An expeditious approach to virus control strategies in tsetse factories (2012)
- Virology, epidemiology and pathology of *Glossina* hytrosavirus, and its control prospects in laboratory colonies of the tsetse fly *Glossina pallidipes* (Diptera: Glossinidae) (2013)

**Writing of project proposal (4.5 ECTS)**

- The salivary secretome of the tsetse fly *Glossina pallidipes* (Diptera: Glossinidae) infected by salivary gland hypertrophy virus (2011)

**Post-graduate courses (3.6 ECTS)**

- Introduction to R for statistical analysis: PE&RC, Wageningen and IAEA, Vienna (2011, 2012)

**Invited review of (unpublished) journal manuscript (2 ECTS)**

- African Journal of Biotechnology: Epidemiology of trypanosome infections (2012)
- Open Veterinary Journal: Tsetse saliva and trypanosome infections (2012)
- Journal of Proteome Research: Proteomics (2013)

**Competence strengthening / skills courses (3.6 ECTS)**

- Scientific writing: Graduate Schools of Wageningen University (2013)
- PhD Competent assessment; Career Centre Meijer & Meijaard (2013)
- Project and time management; PE&RC (2013)
- "Effective behaviour in professional surroundings"; Career Centre Meijer & Meijaard (2013)

**PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)**

- PE&RC Day (2008)
- Introduction weekend (2011)

**Discussion groups / local seminars / other scientific meetings (7.5 ECTS)**

- EPS Autumn school : host-microbe interactomics (2011)
- Seminars in Virology Laboratory, Wageningen (2011-2013)
- Work discussions and seminars at Insect Pest Control Laboratories, Vienna, Austria (2011-2013)
- Virology / Entomology seminar (2012)

**International symposia, workshops and conferences (9 ECTS)**

- International Congress in Invertebrate Pathology and Microbial Control & 44th Annual meeting of the Society for the Invertebrate Pathology; Halifax, Nova Scotia, Canada (2011)
- FAO/IAEA RCM – 3rd annual conference and workshop; Nairobi, Kenya (2011)

- 45th Annual meeting of the SIP; Buenos Aires, Argentina (2012)
- Joint FAO/IAEA RCM – 4th annual conference and workshop; Vienna, Austria (2013)
- ESF/EFS-EMBO Symposium –"Integrated Insect Immunity: From basic biology to environmental applications", Polonia Castle Pultusk, Poland (2013)

**Lecturing / supervision of practical's / tutorials (1.8 ECTS)**

- Molecular Virology PGO (2011, 2012)

**Supervision of an MSc student (3 ECTS)**

Mixed infections of the *Glossina Pallidipes* salivary gland hypertrophy virus in heterologous tsetse fly hosts (2012, 2013).

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**Front cover:** Land area overshadowed by tsetse and trypanosomiasis in sub-Saharan African countries (illustrated by the fly's 'shadow') extending from Mali and Ethiopia in the north and to Angola and South Africa in the south. The map, the tsetse fly and its shadow represents the wider perspective of the PhD thesis: tackling the trypanosomosis menace in sub-Saharan Africa.

**Bottom left corner on the front cover:** Ultra-structural features of *Glossina* hytrosavirus, a double DNA virus that affects laboratory colonies of *G. pallidipes*. The central theme of the thesis was to study the virology, epidemiology and pathology of *Glossina* hytrosavirus, and to develop control strategies in tsetse fly factories.

**Back cover:** An extension of the ultra-structural features of the *Glossina* hytrosavirus.

**Cover layout and graphics design:** H.M. Kariithi, A. Usmani and S. Kermer

**Photo cover:** *Glossina pallidipes* fly photo courtesy of Dr G. Attardo, Yale School of Public Health, New Haven, CT, USA; *Glossina* hytrosavirus TEM images made by Dr J.W.M. van Lent and H.M. Kariithi, Wageningen University Electron Microscopy Centre (WEMC), Wageningen University and Research Centre, Wageningen, The Netherlands.

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