CHAPTER 3.3.

MANAGING PATHOGENS IN INSECT MASS-REARING FOR THE STERILE INSECT TECHNIQUE, WITH THE TSETSE FLY SALIVARY GLAND HYPERTROPHY VIRUS AS AN EXAMPLE

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SUMMARY

Effective management of insect pathogens in insect mass-rearing is fundamental to the successful implementation of area-wide integrated pest management programmes (AW-IPM) with a sterile insect technique (SIT) component. Insect pathogens, notably viral pathogens, affect the sustainable production of high-quality males for the SIT, either by compromising colony establishment and stability or by reducing insect performance, including mating and flight ability. Many pathogenic bacteria, fungi, microsporidia, spiroplasma/mycoplasma and viruses can infect insects. Due to the broad subject of insect pathology, only the pathogens affecting insect mass-rearing facilities producing sterile insects are discussed here, with a special focus on tsetse flies, mosquitoes and codling moth viral pathogens. Special emphasis is given to the successful management of the tsetse salivary gland hypertrophy virus (SGHV), the current management of the codling moth granulovirus (CpGV), and the risk of infections of mass-reared mosquitoes with pathogenic viruses and other vector-borne diseases. Also discussed are the potential risks posed by these pathogens to staff working in mass-production facilities.

1. INTRODUCTION

For centuries, the main goal of organized insect mass-production was to provide reliable and cost-effective sources of high-quality insects for numerous important purposes (Parker, Vreysen et al., this volume). Recent purposes of insect mass-rearing include: (1) release of sexually sterilized males as part of sterile insect technique (SIT) applications (Parker, Mamai et al., this volume), or of genetically modified insects, for the suppression/eradication of target insect pest populations, (2) augmentative releases of parasitoids, predators or pollinators to control target pests or pollinate target crops, (3) application of insects as hosts for the production or dissemination of microbial control agents, (4) use of insects as food and feed (Van Huis 2013; Van Huis et al. 2013; Vantomme et al. 2014; Dossey et al. 2016), and (5) production of economic products such as honey, silk (Needham et al. 1937) and cochineal dyes (Greenfield 2005). Insects are also mass-reared for research and industrial purposes, e.g. the production of chemical components used in cosmetics, medicine for human therapy, and the production of biofuels (Dickerson and Leppla 1992). Additionally, insects are mass-reared for use in celebrations, as pets or objects of interest, and in conservation programmes for endangered or threatened species (Resh and Cardé 2003).

To control insect pests of agricultural or medical importance, various methods including the SIT are used (El-Wakeil and Abdallah 2012). When applied within the concept of area-wide integrated pest management (AW-IPM), the SIT is a proven, effective and environment-friendly tool. This method has been successfully applied to control several insect pests around the world (Goodenough et al. 1983; Klassen et al., this volume). Efficient and effective mass-rearing of the target insect is a fundamental prerequisite when applying the SIT (Dyck 2010). For some purposes, e.g. as hosts for biological control agents or as food and feed sources, mass-rearing of large quantities of the insect is the main objective. However, when producing insects for release into the field where they have to perform a specific task, obtaining high-quality insects is paramount; in the case of the SIT the released sterile males must outcompete wild males in mating with wild females (Parker, Vreysen et al., this volume; Vreysen, this volume). Therefore, high standards must be implemented when producing insects for the SIT. However, microbial agents often compromise insect rearing, representing a major risk and challenge for managers of mass-rearing facilities. Consequently, an essential component to achieve high insect quality is investing sufficiently in expertise and infrastructure to manage effectively and control all potential insect pathogens.

The biological control of insect pests using microbial control agents applied in an augmentative way is a sustainable and environment-friendly tool (Hoffmann and Frodsham 1993; Lacey et al. 2001). Currently, approximately 50 agents, including entomopathogenic viruses, bacteria, fungi and nematodes, are commercially produced; this represents ~1–2% of the global pesticide market (Gallai et al. 2009; Lacey et al. 2015). One of the limitations of using biological control is the mandatory *in vivo* production in the insect hosts of all viruses and some of the other microbial agents. This *in vivo* production system must be highly efficient and economical for the large-scale production of sufficient numbers of the insect hosts, and plays a role in determining the success and the market competence of the biocontrol methods.

In addition to the important role of insect mass-rearing in pest control, in the agricultural sector insects are reared also for other economic reasons. The honeybee *Apis mellifera* L. is important not only for honey production, but also for other products like beeswax (Calderone 2012). More importantly, as the primary plant pollinators, honeybees enhance landscape diversity and are fundamental to the pollination of many agricultural crops, with a worldwide annual value of USD 153 thousand million; in the United States alone the annual value is estimated at USD 17 thousand million (Cohen 2004, 2015; Roeder et al. 2010).

2. MICROBIAL DISEASES AND INSECT MASS-REARING

The microbial contamination of artificial insect diets, particularly by moulds, fungi and bacteria, is a common problem in insect mass-rearing. The rearing conditions for insects are also conducive for microbial growth, especially in the absence of antimicrobial products. For example, Roeder et al. (2010) reported that, in the absence of antifungal agents, within three weeks 40% of the artificial diets used to rear *Heliothis virescens* (F.) larvae were contaminated with microbes. The control of

microbial agents is essential to reduce their negative impact on reared insects and to maintain healthy insect colonies (Alverson and Cohen 2002). Generally, antibacterial and antifungal compounds are ingredients of insect diets to control the microbial contamination. However, many of these compounds can be quite toxic to insects, even at low concentrations (Roeder et al. 2010; Cohen 2015). For example, as the concentration of methylparaben (a common antifungal agent) increases in the diet, it significantly reduces the insect colony's performance (Shikata et al. 1998). Finding the right combination and doses of compounds to fight microbial growth, while at the same time reducing or eliminating any negative effects that these antimicrobial compounds have on the reared insects, can be quite challenging (Cohen 2015).

2.1. Bacterial Diseases

Many types of bacteria can be associated with insects, including saprotrophic facultative parasites and obligate parasites. In insect mass-rearing, many pathogenic bacteria have been reported, but here the focus is on the bacterial diseases found in mass-rearing insects that are used for SIT projects. These insects include various species in the orders Diptera, Lepidoptera, Hymenoptera and Coleoptera.

Bacteria encountered in insectaries are usually innocuous. However, for insects exposed to stressors such as a change from natural to artificial diets, and exposure to elevated temperature and humidity, high density (overcrowding) and toxins, bacteria can multiply extensively within the bodies of insects with a weakened immune system, causing diseases that may be severe or even fatal (Sikorowski and Lawrence 1994). For example, some genotypes of *Bacillus sphaericus* produce insecticidal toxins that are active against mosquito larvae (Boucias and Pendland 1998).

In *Helicoverpa zea* (Boddie), contamination with certain bacterial species, e.g. *Pseudomonas maltophilia* and *Bacillus subtilis*, significantly affects larval mortality, pupal weight, larval and pupal deformation, egg production, and egg hatch. In the absence of antibiotics, *Serratia marcescens*, a highly contagious bacterium, can rapidly bring a moth rearing programme to a virtual standstill. Furthermore, these bacteria can cause sub-lethal infections, significantly compromising insect vigour (Sikorowski and Lawrence 1991).

In fruit flies, some bacteria could cause significant problems. For example, the gram-negative bacteria *S. marcescens* and *P. aeruginosa* can overwhelm Caribbean fruit fly *Anastrepha suspensa* (Loew) larvae and pupae when they are stressed, e.g. exposed to thermal stress by rearing at a temperature >30°C (Greany et al. 1977).

In rearing tsetse flies, bacterial contamination is crucial, and can cause a high abortion rate and high mortalities; this underscores the importance of precautionary measures taken during blood collection and storage, and during the feeding process, including irradiating and freezing blood, and conducting bioassays before exposing colonies to new batches of blood (Feldmann 1994). Although certain species of tsetse flies are more sensitive to bacterial contamination than others, according to Wetzal and Thiemann (1979) *Glossina m. morsitans* Westwood adult flies were more sensitive to *P. aeruginosa* bacterial contamination than adults of *G. p. palpalis*

Robineau-Desvoidy; adults of the latter eliminated a bacterial infection at nine days post-ingestion, but *G. m. morsitans* adults required 14 days to clear the infection.

The best way to avoid bacterial contamination in mass-rearing is to maintain sanitary conditions (Inglis and Sikorowski 2009a, b). When using the membrane feeding technique in tsetse fly mass-rearing, this means applying prophylactic measures, i.e. working in aseptic conditions with sterile feeding membranes, aluminium trays and blood. Using sanitation protocols when rearing the Caribbean fruit fly *A. suspensa* avoided the negative impact of bacterial contamination with *S. marcescens* and *P. aeruginosa* (Greany et al. 1977). As a precaution, mass-rearing facilities often include either antibiotics, e.g. chlortetracycline, antifungals, e.g. nipagin and benzoic acid, or formalin as a general antimicrobial in adult and larval diets (Cohen 2015).

When sanitation is practised and antibiotics are available, bacterial contamination in insect mass-rearing is rare. However, it is important to note that prolonged application of antibiotics may lead to resistant bacterial strains. The wise use of antibiotics is not a substitute for, but a complement to, good sanitation for disease control (Sikorowski 1984).

2.2. Fungal Diseases

There are many species of fungi that infect insects, e.g. species in the genera *Aspergillus*, *Beauveria*, *Entomophaga*, *Entomophthora*, *Hirsutella*, *Metarhizium*, *Nomuraea*, *Paecilomyces* and *Penicilium*. Species of *Entomophthora* have a worldwide distribution, and play effective roles in controlling many insects of economic importance in the orders Hemiptera, Homoptera, Diptera, Lepidoptera, Coleoptera, Orthoptera and Hymenoptera (MacLeod 1963; MacLeod and Muller-Kogler 1973). Fungi are unique among insect pathogens in that they infect their hosts primarily through the cuticle, although a few taxa may invade through the host's alimentary canal. By simple contact, dissemination of the infection among the individuals of a colony occurs rapidly. Due to the spread of fungal spores by wind and rain, fungal diseases can occur in field-collected insects. This scenario was reported for grasshoppers, where *B. bassiana* caused 90% mortality (Inglis and Sikorowski 2009b).

In general, fungus infection is humidity-dependent, a condition which is usually found in large-scale insect mass-production facilities where artificial diets are used. Furthermore, holding large numbers of insects in a limited space (for economic or convenience reasons) facilitates the spread of fungal diseases. In addition, many saprophytic species such as *Aspergillus* and *Penicilium* spp. growing on the artificial diet might be facultative pathogens, requiring the use of an antifungal compound to avoid infection. The use of fungicides to control fungal infections is a prerequisite for mass-rearing some insect species.

Some fungi such as *Aspergillus flavus* produce aflatoxins, among the most potent carcinogenic compounds; this can be a major health issue for workers in mass-rearing facilities. Also, fungi are health risks -- breathing hazards and possible contamination via the skin, eyes, and nasal passages (Burgner et al. 1998; Revankar et al. 1999; Butt et al. 2001).

2.3. Protozoan, Microsporidia and Mycoplasma/Spiroplasma Diseases

Most of the entomopathogenic protozoa and microsporidia (thought to have evolved from a fungus (Keeling et al. 2000)) produce chronic, non-lethal infections that reduce the host reproduction (Hurd 1993). These pathogens also cause irregular growth, loss of appetite and malformed larvae, pupae or adults with reduced vigour, fecundity and host lifespan. Although microsporidia have been reported to infect all insect orders, the majority of genera occur in dipteran hosts, which represent an important challenge when applying the SIT to insect disease vectors such as tsetse flies and mosquitoes, and to fruit flies. Microsporidia infection can occur in three different ways: (1) *per os*, through feeding on spore-contaminated food or on dead or moribund insects, (2) transovarially from a female to her offspring on or in eggs (which includes newly hatched larvae feeding on the egg chorion), and (3) directly through the insect cuticle. In certain cases, e.g. *Helicoverpa zea*, all offspring of microsporidia-infected females are infected. In addition, in a lepidopteran insectary, microsporidia spores may be disseminated via moth scales. Once ingested by an insect host, microsporidia spores germinate in the midgut and infect midgut tissue and hemocytes. The severity of the infection is dependent on the number of ingested spores, temperature and larval age. Ingestion of a large number of spores usually causes mechanical damage to the peritrophic membrane and midgut epithelium, thus allowing the ingress of bacteria present in the gut lumen. In such cases, the infected insects soon die. Although the ingestion of small numbers of spores does not kill the host insect, this causes a chronic infection (Inglis and Sikorowski 2009b).

2.4. Entomopathogenic Nematodes

Infection with entomopathogenic nematodes in mass-rearing environments is a rare event. However, new insect colonies established using field-collected insects might be infected by nematodes. Although there are about thirty families of nematodes associated with insects, the majority of these are animal and plant parasites and use insects only as vectors. Nematodes that infect insects as primary hosts occur in seven families. Nematode infection causes sterility, reduced fecundity, development delay, aberrant behaviour, colour changes and host death. Most of the time the entomopathogenic nematodes do not reach adulthood in the hemocoel of the infected host. Rather, such nematodes emerge from the host as post-parasitic juveniles, moult to the adult stage, mate, and produce offspring (Kaya and Stock 1997). Infective juveniles directly penetrate the host's exterior integument using their stylets. Nematodes from the families *Steinernematidae* and *Heterorhabditidae* are obligate parasites of insects and carry mutualistic bacteria of the genus *Xenorhabdus* within their guts; once the nematodes gain access to the hemocoel, the bacteria are released into the hemocoel and kill the host within 24 hours (Kaya and Stock 1997).

2.5. Viral Diseases in Insect Mass-Rearing

Viruses are among the agents that have a severe pathogenic impact on insect populations, both in the field and the laboratory. Viruses have been found to be responsible for epizootics in mass-reared useful insects, e.g. silkworm (Watanabe 1986), crickets (Szelei et al. 2011; Weissman et al. 2012) and honeybee (Chen and Siede 2007; Wilfert et al. 2016). Insects can be infected with DNA or RNA viruses. Of the approximately 100 families of viruses infecting eukaryotic organisms (ICTV 2017), 16 families of RNA viruses (Ryabov 2017) and 12 families of DNA viruses (Tijssen et al. 2016; Williams et al. 2017) infect several important orders of insects, e.g. Diptera, Lepidoptera, Hymenoptera, Coleoptera and Orthoptera. Some of these viruses are specific to arthropods (largely insects), e.g. families Dicistroviridae and Iflaviridae encompassing small RNA viruses, or families Baculoviridae, Nudiviridae and Ascoviridae encompassing large DNA viruses. Other RNA viruses (Nodaviridae and Reoviridae) or DNA viruses (Iridoviridae, Parvoviridae and Poxviridae) consist of genera, e.g. *Alphanodavirus*, *Cypovirus* and *Chloriridovirus*, or subfamilies, e.g. *Densovirinae* and *Entomopoxvirinae*, specifically pathogenic to insects, whereas other genera or subfamilies of these families infect vertebrates or humans but not insects. The honeybee is infected by at least 12 viruses belonging to five different virus families. In addition, some arthropod-borne viruses replicate both in their insect hosts and their vertebrate hosts. Examples include members of the virus families Bunyaviridae and Flaviviridae (Ryabov 2017).

Viral infections in insect populations can be symptomatic (with overt disease symptoms) or asymptomatic (hidden symptoms). While the former can result in the loss of fitness or death of the infected insects, the latter seems usually not to have fitness costs to the host. Cryptic latent virus infections are frequently observed in wild insect populations as well as in small- and large-scale laboratory colonies. Under certain unfavourable abiotic conditions, e.g. temperature and humidity, or biotic stress, e.g. increase in population density, shortage or change of food or accidental contamination of food, these hidden viral infections can be activated and soon result in outbreaks of epizootic infections (Tanada 1963; Weissman et al. 2012). However, the most frequent origin of viral outbreaks is the accidental introduction into laboratory colonies of individuals from other colonies (or collected from the field) that carry asymptomatic viral infections.

Until the discovery of several small RNA viruses in *Drosophila* species, several laboratories that use *Drosophila* as a model for genetic research have been working for years with colonies carrying hidden virus infections (Jousset et al. 1972; Teninges and Plus 1972; Plus et al. 1975). Further, the development of deep sequencing and metagenomic analysis tools has led to the rapid discovery of novel virus strains in asymptotically infected insects. Examples include the honeybee filamentous virus with a genome of circular dsDNA of ca. 500 kbp in size (Gauthier et al. 2015), the small circular ssDNA of ca. 2 kb viruses (family Circoviridae) isolated from crickets, dragonflies and mosquitoes (Rosario et al. 2012; Pham et al. 2013; Garigliany et al. 2015), rhabdoviruses (Longdon et al. 2015) and endogenous retroviruses (Akkouche et al. 2012; Pelisson et al. 2002) of *Drosophilidae*. Hidden latent viral infections represent a major challenge for insect mass-rearing for the SIT because rearing for many generations under a high density might represent a stress

factor compromising the insect immune system and leading to the emergence of latent viral infections.

2.5.1. *Viral Diseases in Lepidoptera*

Several lepidopteran species of economic importance are the targets in SIT projects (e.g. codling moth, false codling moth *Thaumatotibia leucotreta* (Meyrick), pink bollworm *Pectinophora gossypiella* (Saunders) and cactus moth *Cactoblastis cactorum* (Berg)) or the basis of industrial silk production (silkworm *Bombyx mori* (L)). Insects belonging to the order Lepidoptera are the hosts of many insect viruses, notably in the larval stages. Outbreaks of epizootics caused by viral agents of the family Baculoviridae were frequently reported to control natural dense populations of lepidopteran pests (Cory and Bishop 1997; Cory et al. 1997; Moscardi 1999; Fuxa 2004). Owing to their safety for humans and specificity in host range, usually limited to a single or a limited number of insects of the same species, these viruses are used for the biological control of several crop and fruit pests. On the other hand, baculoviruses represent a major problem for the mass-rearing of Lepidoptera used in SIT projects, e.g. the codling moth (Tanada 1964) or the silkworm (Summers 2006). More than 160 years ago the first insect viral infection, (i.e. baculovirus in the silkworm), was reported: in 1856 Cornalia and Maestri were able to associate the refractile occlusion bodies (now known as polyhedral) with the disease in silkworm (Summers 2006). The granulovirus CpGV in the codling moth was first reported in Mexico in 1964 (Tanada 1964). Currently, CpGV is widely used as a biological control agent to control this pest. On the other hand, strong measures to control CpGV are required to avoid a severe negative impact on the stability of the codling moth colony used for the SIT in the Okanagan Valley of British Columbia, Canada (section 3.2.). In addition to these two viruses, similar baculovirus infections have been reported in many other lepidopteran insects.

Densoviruses represent another major threat for the mass-rearing of Lepidoptera. These viruses are highly pathogenic to their hosts; they are responsible for epizootics in natural populations of some major lepidopteran pests, e.g. oil palm defoliators and sugar cane and corn borers, and in mass-reared insects used as pet food, e.g. crickets and the greater wax moth *Galleria mellonella* (L.) (Fédière 2000; Bergoin and Tijssen 2010). The *Bombyx mori* densovirus (BmDV-1) is the causative agent of the infectious flacherie of the silkworm (Shimizu 1975), one of the most deleterious diseases in sericultural farms. As a result of a demonstration that the susceptibility of the silkworm to BmDV-1 varied from one strain to another, it was possible to select resistant strains (Eguchi et al. 1991).

2.5.2. *Viral Diseases in Tsetse Flies*

In 1987 the first collapse of the *Glossina pallidipes* Austen colony, established at the FAO/IAEA Insect Pest Control Laboratory (IPCL) in Seibersdorf, Austria, occurred; it was caused by the salivary gland hypertrophy virus (SGHV) (Fig. 1). This colony was initiated from various batches (528 pupae) collected from the Lambwe Valley, Kenya, in November 1983. The colony was maintained and increased to a thousand productive females, but thereafter the colony stagnated at that level, having a low fly

emergence rate, high daily mortality (1.8% in flies older than 50 days), low fecundity (<1.1 pupae per female per month) and a low insemination rate (<85%). Microscopic examination of dissected flies revealed that up to 85% of males and 70% of females had hyperplastic salivary glands (Fig. 1). Males with hyperplastic salivary glands showed testicular degeneration and aspermia. These problems led to discontinuation of the colony in 1988 (FAO/IAEA 1987, 1988). Thereafter, a new *G. pallidipes* colony was established with insects originating in Tororo, Uganda, and donated by the Tsetse Research Laboratory, Bristol, England. However, the new colony was established and successfully maintained without an investigation of the cause of the collapse of the previous colony (FAO/IAEA 2006).

Figure 1. Symptoms of *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV) in tsetse fly *G. pallidipes* males. (A) Males with asymptomatic (left) and symptomatic (right) infection (reproduced from Abd-Alla et al. 2009a, with permission from Elsevier); (B) Tsetse males with normal salivary glands (NSG) and hypertrophied salivary glands (HSG) (reproduced from Abd-Alla et al. 2013a, with permission from Elsevier); (C) Transmission electron microscopy (TEM) micrograph of GpSGHV virus particles (right), cross section (left). The TEM images were made by J. van Lent and H. M. Kariithi (Wageningen University, reproduced with permission).

Following pest suppression and the release of sterile males, in 1997 the tsetse fly *G. austeni* Newstead was eradicated on the island of Unguja, United Republic of Tanzania (Vreysen et al. 2000; Hendrichs et al. 2007). This success prompted other African countries to consider applying the SIT to eradicate tsetse fly populations. A programme to create a zone free of *Glossina pallidipes* in the Southern Rift Valley of Ethiopia was initiated in 1996 (Vreysen et al. 2000; Feldmann et al., this volume).

This project stimulated the IPCL to conduct research on the problematic *G. pallidipes* colony. Since the available colony was initiated from insects collected in Uganda, it was decided that a *G. pallidipes* colony should be started using flies from Ethiopia. Therefore, a new *G. pallidipes* colony was initiated in 1999 with pupae collected in Arba Minch, Ethiopia. This colony became well established, and in 2001 it reached about 15 000 productive females.

However, similar to the 1987 colony, the new colony declined steadily over a 2-year period, becoming extinct in 2002. Similarly, dissections revealed that up to 85% of both male and female flies had salivary gland hypertrophy (SGH), a syndrome first described in wild populations of *G. pallidipes* (Table 1) (Abd-Alla et al. 2011). To overcome the problems of managing a *G. pallidipes* colony, research was conducted for more than ten years, resulting in effective management packages (section 3.1.).

Symptoms of the SGH were initially reported in 1932 (Table 1). During dissection of *Glossina pallidipes* specimens to observe trypanosomes in Zululand, South Africa, swollen salivary glands were observed (Whitnall 1932; Whitnall 1934). Later, the SGH symptoms were reported in *G. morsitans* (Burt 1945). In the 1970s, the SGH syndrome was associated with a virus found in cytoplasmic vacuoles of the salivary glands and midgut epithelial cells of *G. fuscipes* Newstead and *G. morsitans* (Jenni 1973; Jenni and Steiger 1974a, b; Jenni and Böhlinger 1976). At that time the virus was described as “virus-like particles” (VLPs), and erroneously thought to be an arbovirus.

The first link between the SGH and the salivary gland hypertrophy virus was reported by Jaenson (1978) who examined the hypertrophied salivary gland in *G. pallidipes* from Kenya using electron microscopy; it was found that rod-shaped virus particles were present only in salivary glands that showed this syndrome. The particles were absent in non-hypertrophied salivary glands. Based on the assumption that the new virus might be a potential biological control agent against tsetse flies, many researchers made a concerted effort to explore this idea. Subsequently, SGH syndromes were reported in many tsetse species, e.g. *Glossina austeni*, *G. m. morsitans*, *G. nigrofusca nigrofusca* Newstead and *G. pallicera pallicera* Bigot (Burt 1945; Ellis and Maudlin 1987; Gouteux 1987). Although containing a double-stranded DNA genome, the virion structure did not suggest that the tsetse virus should be assigned to any of the existing insect virus groups (Burt 1945; Odindo et al. 1986; Ellis and Maudlin 1987; Gouteux 1987). Pathological effects noted in various countries included sex distortion and a reduction in insemination rates, fecundity and lifespan (Jaenson 1986).

The main research question was to explain why the Ethiopian-derived colony collapsed, unlike the Ugandan-derived colony from Tororo which was, and still is, sustainable. Notably, the prevalence of the SGH syndrome was about 10% in the Tororo colony compared with 85% in the Arba Minch colony. This significant difference in the prevalence of the SGH syndrome raised the question whether these colonies were infected by the same or different virus strains. Therefore, the complete genome of the virus isolated from the Tororo colony was sequenced and annotated. The sequencing data identified the genome of the virus as a circular double-stranded DNA molecule over 300 kb in size; this confirmed the previous observation of Odindo (1986) that this virus is unique and could not be assigned to any existing

insect virus family. Therefore, a new family of insect viruses called Hytrosaviridae was created, encompassing the tsetse fly virus and a similar virus from the house fly *M. domestica* as two members of this family. The name Hytrosaviridae was derived from Hytrosa, sigla from the Greek '*Hypertrophia*' for '*hypertrophy*' and '*sialoadenitis*' for 'salivary gland inflammation' (Jaenson 1986; Abd-Alla et al. 2008, 2009b; Garcia-Maruniak et al. 2009; Lietze et al. 2011). The recent sequencing and annotation of the genome of the Ethiopian GpSGHV (GpSHGV-Eth) strain revealed deletions/insertions in 37 ORFs and 17 ORF deletions and 24 novel ORFs compared with the Ugandan GpSGHV, which might explain the differential pathogenesis between the two colonies (Abd-Alla et al. 2016).

In addition, it was demonstrated that the GpSGHV infects other tsetse species, and causes a negative impact on the flies' performance without developing salivary hypertrophy (Demirbas-Uzel et al. 2018a, b). Also, it was reported that the virus has different strains/genotypes circulating in the wild tsetse population, and the virus infection affects the flies' immune system (Kariithi et al. 2018a, b; Meki et al. 2018a, b, c).

The sequence data of the SGHV isolated from the Tororo-derived (Uganda) *G. pallidipes* represents the reference virus genome; the virus is named as the GpSGHV-Uga. The availability of the GpSGHV-Uga genomic data enabled the development of virus detection tools such as a conventional, non-destructive PCR and a quantitative PCR (qPCR) which were used to diagnose and quantify the viral infections in tsetse colonies. The PCR detection method revealed that, although only about 10% of the Tororo colony exhibited SGH symptoms as an overt infection, almost all flies harboured the virus infection as an asymptomatic infection.

Quantitative PCR provided further evidence that the virus titre unequivocally correlated with the SGH symptoms, thus providing additional proof that the GpSGHV causes SGH. Sequence analysis of the SGHV-Uga genome revealed that it shared several gene similarities with other large DNA viruses such as baculoviruses, nudiviruses or entomopoxviruses. Of particular interest was the similarity of the SGHV DNA polymerase (a gene responsible for replicating the DNA) with that of the human herpes virus. This similarity led to the use of the antiviral drugs commercially available against herpes virus infection to control the SGHV infection (section 3.1.1.) (Abd-Alla et al. 2008, 2009b, 2014).

The average low rate (0.5–1%) of virus infection with overt SGH symptoms or covert latent infection prevalence in tsetse wild populations significantly differed from the laboratory colonies; in the laboratory a covert infection occurred in almost all flies (Odindo 1982; Odindo and Amutalla 1986; Abd-Alla et al. 2007, 2009a). To explain this difference our attention was drawn to the *in vitro* feeding system which was developed at the IPCL in Seibersdorf, and is currently used in all tsetse mass-rearing facilities, i.e. flies (in holding cages) are fed in several successive rounds on the same membranes containing defibrinated blood. The detection of infectious SGHV in the blood remaining on the membranes after feeding flies infected with the SGHV clearly demonstrated that the successive feeding rounds significantly favoured virus transmission within the tsetse colony (Fig. 2) (Feldmann 1994; Abd-Alla et al. 2010, 2011, 2013b). This important finding was the basis for modifying the feeding regime to manage the SGHV infection (section 3.1.2.).

Table 1. Chronological history of the discovery and distribution of SGHVs¹

Major contribution	Reference
<ul style="list-style-type: none"> • First published record of SGH in <i>Glossina</i> spp. • Suggestion that SGH is sex-linked • Description of virus particles in <i>G. morsitans</i> and <i>G. fuscipes fuscipes</i>; suggestion of Golgi-ER viral assembly 	Whitnall 1932, 1934 Burt 1945 Jenni 1973; Jenni and Steiger 1974a, b; Jenni and Böhringer 1976
<ul style="list-style-type: none"> • First published record of SGH in <i>Merodon equestris</i> F. • First clear association of viral particles with SGH • SGH in <i>M. equestris</i> • Demonstration that viral particles are infectious <i>per os</i>; first report that <i>Glossina</i> virus has dsDNA genome 	Lyon and Sabatier 1973 Jaenson 1978 Amargier et al. 1979 Odindo 1982, 1988; Odindo and Amutalla 1986; Odindo et al. 1986
<ul style="list-style-type: none"> • First report on reduced insemination rates, fecundity and lifespan in laboratory colonies of <i>G. pallidipes</i> • SGH in wild tsetse from Zimbabwe and Côte d'Ivoire 	Jaenson 1986 Ellis and Maudlin 1987; Gouteux 1987
<ul style="list-style-type: none"> • Poor productivity of <i>G. pallidipes</i> colonies at FAO/IAEA, Austria • Proposal of <i>Glossina</i> virus as a biocontrol agent • Demonstrated transmission of <i>Glossina</i> virus after artificial infection 	FAO/IAEA 1987, 1988 Odindo 1988
<ul style="list-style-type: none"> • Cytopathology of virus particles in tsetse salivary glands • SGH in <i>G. m. swynnertoni</i> Austen and <i>G. brevipalpis</i> Newstead 	Jura et al. 1988, 1989, 1993; Jura and Davies-Cole 1992 Kokwaro et al. 1990, 1991
<ul style="list-style-type: none"> • First published record of SGH in <i>Musca domestica</i> L. • SGHV in tsetse milk glands, midgut and male accessory reproductive glands • Collapse of an Ethiopian-derived <i>G. pallidipes</i> colony at IPCL, Seibersdorf, Austria 	Shaw and Moloo 1993 Coler et al. 1993 Sang et al. 1996, 1997, 1998, 1999)
<ul style="list-style-type: none"> • Virus particles in male accessory reproductive glands of <i>G. m. morsitans</i> 	FAO/IAEA 2002
<ul style="list-style-type: none"> • <i>G. pallidipes</i> SGHVs genome sequenced • <i>M. domestica</i> SGHVs genome sequenced • Establishment of Hytrosaviridae family • Transcription analysis of <i>M. domestica</i> SGHV • Description of proteome, ultrastructure and morphogenesis of <i>Glossina</i> virus 	Kokwaro 2006 Abd-Alla et al. 2008 Garcia-Maruniak et al. 2008 Abd-Alla et al. 2009b Salem et al. 2009
<ul style="list-style-type: none"> • World-wide distribution of SGHV in <i>M. domestica</i> • SGHV-like virus described in accessory gland filaments of the parasitic wasp <i>D. longicauda</i> (Ashmead) 	Kariithi et al. 2010, 2013a Prompiboon et al. 2010
<ul style="list-style-type: none"> • Negative impact of antiviral drug (valacyclovir) on SGHV replication in tsetse • Role of endosymbionts on transgenerational transmission of SGHV in <i>G. pallidipes</i> 	Luo and Zeng 2010 Abd-Alla et al. 2012
<ul style="list-style-type: none"> • Successful management of <i>Glossina</i> hytrosavirus in <i>G. pallidipes</i> colonies using modified feeding system • Successful management of <i>Glossina</i> hytrosavirus and eradication of SGH in <i>G. pallidipes</i> colonies using combination of antiviral drugs and modified feeding system 	Boucias et al. 2013 Abd-Alla et al. 2013b
<ul style="list-style-type: none"> • Description of molecular pathways modulated by GpSGHV in <i>G. pallidipes</i> and <i>G. m. morsitans</i> • Genome of <i>G. pallidipes</i> SGHV (Ethiopia) sequenced 	Abd-Alla et al. 2014 Kariithi et al. 2016 Abd-Alla et al. 2016

¹Table adapted from Kariithi 2013, used with permission

Figure 2. Horizontal transmission of SGHV in *G. pallidipes*. (A) In vitro membrane feeding used in tsetse mass-rearing; (B) Simulation of the in vivo feeding on live animals. Red, flies with SGH; black, uninfected flies (Abd-Alla et al. 2011).

2.5.3. Viral Diseases in Mosquitoes

Viruses Pathogenic to Mosquitoes. There are several types of viral pathogens that cause diseases in mosquitoes, most of which belong to four major groups: (1) the baculoviruses (NPVs) (Baculoviridae: Nucleopolyhedrovirus) (the most common mosquito viruses), (2) cytoplasmic polyhedrosis viruses (CPVs) (Reoviridae: Cypovirus), (3) densoviruses (DVs) (Parvoviridae: Brevidensovirus) and (4) the iridoviruses (MIVs) (Iridoviridae: Chloriridovirus). Baculoviruses, densoviruses and iridoviruses are DNA viruses, while cypoviruses are the main RNA viruses in mosquitoes (Becnel 2006; Becnel and White 2007; Kariithi 2013; Tijssen et al. 2016).

The first reported mosquito baculovirus (AesoNPV) was isolated from *Aedes sollicitans* (Walker) in Louisiana in 1969, and over the next two decades, NPVs were isolated from 10 additional mosquito species in the genera *Aedes*, *Anopheles*, *Culex*, *Psorophora*, *Uranotenia* and *Wyeomyia* (Afonso et al. 2001). Baculoviruses are highly pathogenic, and are responsible for epizootics in field populations of mosquito larvae, e.g. the *Culex nigripalpus* Theobald nucleopolyhedrosis virus CuniNPV (Afonso et al. 2001). Moreover, a baculovirus infection can be destructive to mass-reared mosquito colonies.

The highly pathogenic mosquito densoviruses (MDVs) are non-enveloped and relatively stable in the field or under laboratory conditions, making it very difficult to inactivate them. The MDVs are highly specific to mosquitoes in aquatic larval habitats. Larvae that survived an MDV infection and developed to the adult stage exhibit a dose-dependent shortening of their adult lifespan. Infected females vertically transmit the virus to their offspring at the new oviposition site. These characteristics make the MDVs suitable biocontrol agents against mosquito populations. In contrast, when densoviral diseases spread in laboratory colonies, these same characteristics usually cause control efforts to be unsuccessful. In most cases the colony has to be destroyed, and equipment and the facility decontaminated (Carlson et al. 2006). MDV infections can be latent, e.g. the *Aedes albopictus* densovirus chronically infecting an apparently healthy subclone of an *Aedes albopictus* C6/36 cell line; this was fortuitously discovered to be highly pathogenic by feeding *Aedes aegypti* (L.) larvae in C6/36 cell flasks (Jousset et al. 1993).

In addition to MDVs, mosquito infection with several other viruses has been reported. For example, recent deep sequencing of samples derived from three colonies of *Anopheles* spp. maintained at the Institute Pasteur (Carissimo et al. 2016) led to the identification of two “novel” RNA viruses, a cytoplasmic polyhedrosis virus (multisegmented dsRNA) and a cripavirus (bisegmented ssRNA) that chronically infected these colonies. A new group of ssRNA viruses with a large genome (20 kb), classified in the family Mesoniviridae, has been identified recently in different species of mosquitoes in the genera *Culex* and *Anopheles* in Côte d’Ivoire (Zirkel et al. 2011, 2013) and in *Culex vishnui* Theobald and *C. taeniorhynchus* Wiedemann in Vietnam (Nga et al. 2011).

Vector-Borne Diseases and Risk of Infection to Staff. The mosquito species currently reared in laboratory colonies, including for the SIT, especially in large-scale factories, belong to three genera of culicids: *Aedes*, *Anopheles* and *Culex*. These species encompass the vectors of the major human parasitic diseases, e.g. malaria or flavivirus arboviral pathogens such as yellow fever, West Nile encephalitis, dengue, Chikungunya and Zika viruses. The vertical transmission of several of these viruses in their natural mosquito vectors has been demonstrated experimentally under laboratory conditions and detected in wild populations of mosquitoes (Bolling et al. 2015; Tesh et al. 2016; Thangamani et al. 2016).

Recent investigations on the mosquito microbiome revealed that, in addition to these classical arboviral pathogens, mosquitoes house diverse varieties of endosymbiotic RNA viruses, many of which are phylogenetically related to human pathogenic arboviruses in the families Flaviviridae, Bunyaviridae and Togaviridae (Cook et al. 2013; Marklewitz et al. 2013; Huhtamo et al. 2014; Bolling et al. 2015; Li et al. 2015). In contrast to classical arboviruses, which replicate in their invertebrate and vertebrate hosts, these viruses are incapable of replicating in vertebrate cells, and are designated as insect-specific viruses (ISVs). Several of these ISVs are vertically transmitted (Saiyasombat et al. 2011; Bolling et al. 2012; Haddow et al. 2013), strongly suggesting their long-term association with their insect host. Experimental data have provided evidence that ISVs can alter the mosquito’s susceptibility to certain pathogenic arboviruses (Kent et al. 2010;

Bolling et al. 2012; Hobson-Peters et al. 2013; Kenney and Mertz 2014). These data have opened up new perspectives for the development of mosquito strains refractory to the transmission of vertebrate arboviruses.

Taken together, these data imply a risk to staff in mass-rearing mosquitoes – they might become infected with mosquito-borne diseases. Therefore, strict measures must be followed to avoid the introduction of infected mosquitoes into colonies, or exposure of infected humans to the mosquito colonies. In this respect, field-collected mosquito samples should first be quarantined and diagnosed for virus infections using sensitive diagnostic tools, e.g. by qPCR, prior to the introduction of such mosquitoes into the existing fly colonies or in the establishment of new colonies. To protect staff from infections with mosquito-vectored viruses, protective measures must be undertaken, e.g. minimizing and eliminating escapees, wearing protective clothing and adhering to control measures (ASTMH 2001). Personal protection is especially important in the event that mosquitoes escape from rearing cages. Moreover, strict hygiene in the environment around mass-rearing facilities is important to prevent escaped mosquitoes from finding breeding places; this also minimizes the chance of staff becoming infected with a virus and the reintroduction of a virus into the colonies. An additional risk in respect to mass-reared mosquito colonies is the emergence of a latent virus infection, especially under stressful rearing conditions, e.g. high densities, which might compromise the mosquito immune system. Therefore, to ensure colony safety and sustainability, it is recommended that mosquito colonies be surveyed routinely for both pathogenic and mosquito-borne viruses.

2.5.4. Viral Diseases in Reared Fruit Flies and Wild Populations

As in mosquitoes (section 2.5.3.), several viruses have been identified in fruit flies (Webster et al. 2015). In addition to the covert infections of laboratory or wild strains of *Drosophilidae* (section 2.5.), several RNA viruses have been isolated from species of *Tephritidae* belonging to two genera of major economic interest – *Ceratitis* and *Bactrocera* (*Dacus*). Plus et al. (1981) isolated a reovirus and a picorna-like virus from the J. C. R. Ispra strain of *Ceratitis capitata* (Wiedemann). Bashiruddin et al. (1988) found a picornavirus in various life stages of the Queensland fruit fly *Bactrocera tryoni* (Froggatt) in a laboratory colony; the virus induced premature mortality and decreased fecundity in infected flies.

Pathogenicity tests using various insect viruses demonstrated the sensitivity of the olive fruit fly *Bactrocera oleae* (Rossi) to *per os* infection with the picornavirus Cricket Paralysis Virus (CrPV). Feeding the flies on a solution containing the virus resulted in 50% mortality in 5 days and more than 80% mortality within 12 days. The virus was found to be horizontally transmitted from infected to healthy flies by fecal contamination. The flies could also be infected with the type 21 iridovirus from *Helicoverpa armigera* (Hübner) (Manousis and Moore 1987). A virus exhibiting the salient structural and genomic features of reovirus virions was isolated from wild *B. oleae* flies from Greece. This virus actively replicated in midgut cells where it aggregated in cytoplasmic paracrystalline arrays, and was abundantly released to the gut lumen through the microvilli (Anagnou-Veroniki et al. 1997).

The Mediterranean fruit fly *C. capitata* was reported to be infected with the

Drosophila melanogaster Meigen C picornavirus (DCV); the infection resulted in increased virus titre and fly mortality (Jousset 1976). Recently, three novel picornaviruses infecting *C. capitata* were discovered and named as *C. capitata* iflavivirus 1 and 2 (CcaIV1 and CcaIV2) and *C. capitata* noravirus (CcaNV). The CcaIV2 and CcaNV infections were abundant in most of the fly strains used in the various SIT projects around the world, as well as in field captures in the east of Spain. Although there is no direct proof that the virus infection affects the fly's performance, high viral titres of CcaNV were associated with a reduction in the lifespan of males released into the field for the control of this pest. Taken together, these data strongly suggest the possibility that CcaNV impairs the fitness of the sterile flies (Llopis-Gimenez et al. 2017).

In addition to tephritid fruit flies, several laboratory or wild strains of Drosophilidae have been reported to be infected covertly with many RNA viruses, e.g. the DCV (Arnold et al. 2015), the Sigma virus (Rittschof et al. 2013), the Nora virus (Cordes et al. 2013) and the *Drosophila* P virus (Plus and Duthoit 1969; Teninges and Plus 1972). Further, the spotted-wing drosophila *Drosophila suzukii* (Matsumura) was reported to be infected with *Drosophila* C virus and Flock House virus (Cattell et al. 2016).

2.5.5. Viral Diseases in Honeybees, Silkworms and Crickets

Historically, honeybees and silkworms are the most important insects used in the production of economic products. Many diseases, including viral diseases, were first recorded in these insects (Trager 1935; Glaser and Stanley 1943). However, the viral diseases in honeybees and silkworms are not discussed here; they have been well documented by others (Aruga 1971; Cox-Foster et al. 2007; vanEngelsdorp et al. 2009; Francis et al. 2013; Jiang and Xia 2014).

Recently, the intensive mass-production of field crickets as pet food resulted in the presence of viral diseases, causing enormous losses and requiring urgent intervention (Weissman et al. 2012). The first epizootic of the European house cricket *Acheta domesticus* (L.), with a densovirus (AdDV) as the causative agent, was detected in a Swiss commercial factory (Meynadier et al. 1977). Several other outbreaks have been observed in Europe, but it was only in 1991 that an AdDV epizootic was reported in a cricket farm in south-eastern USA (Styer and Hamm 1991). In 2009/2010 severe AdDV outbreaks in cricket farms decimated the multimillion dollar cricket pet food industry all over North America, from Quebec to Alberta and Florida to California (Weissman et al. 2012). Sequence analyses of the viral genome revealed a 99% common identity between the 1977 European isolate and different 2009 North American isolates. This finding demonstrated the very high genetic stability of the virus, and provided evidence that these epizootics were not due to the emergence of novel virulent strains of the virus (Szelei et al. 2011).

3. STRATEGIES TO MANAGE PATHOGENS IN INSECT MASS-REARING

Some of the viruses, bacteria, protozoa and fungi found in insect mass-rearing are known disease-causing pathogens, but others have no known host-fitness costs. Pathogens may be introduced into mass-rearing facilities from the field-collected

insects used to establish colonies, or could be latent and erupt as a result of intense and continuous rearing under stressful rearing conditions in the facilities. Some of the conventional pathogen management techniques in insect mass-rearing include isolation and rearing of healthy individuals from colonies that manifest mixed infections, changing colony environmental conditions and the application of antibiotics. Although these techniques are useful in the management of disease outbreaks, their effectiveness is mostly short-lived. Effective pathogen management largely depends on the type, transmission mode, and persistence of the pathogens as well as the manifestations of the disease.

The next five sections highlight the effective management of salivary gland hypertrophy syndrome (SGH) in mass-rearing tsetse flies. Also discussed are different approaches and techniques that are used in the management of viral and non-viral diseases in mass-rearing codling moths, honeybees, and silkworms.

3.1. Combined Approaches to Manage SGHV in Tsetse Mass-Rearing

Outbreaks of the SGH syndrome in tsetse mass-rearing facilities are a rare occurrence in view that SGHV presence is mostly asymptomatic. However, when the SGH occurs, the fecundity of some tsetse species, especially *G. pallidipes*, is compromised. Except in *G. pallidipes* colonies, SGHV infections are mostly asymptomatic, but unknown factors, such as environmental stressors and perhaps host physiology, can promote SGH outbreaks, resulting in the loss of colony productivity and the decline of an entire tsetse colony over several generations (section 2.5.2.). The mechanisms by which SGHV infections persist in tsetse colonies are known partially. However, it is clear from various experiments and observations that the virus is vertically transmitted and persists in subsequent fly generations (Boucias et al. 2013). Horizontal SGHV transmission is well documented (Abd-Alla et al. 2010), and is the principal route through which viral infections spread in tsetse mass-rearing (Fig. 2). Therefore, it is important to minimize the risk of SGH outbreaks, which can be done through several approaches, as described below.

3.1.1. Antiviral Drugs

For viruses that are intermittently reactivated from latency to symptomatic disease manifestations, antiviral drugs can be administered to keep the virus infection in check. Studies in human herpesviruses (HHVs, family Herpesviridae) have shown that the administration of antiviral drugs such as acyclovir and valacyclovir reduced the prevalence, titre and shedding of the virus from infected host cells (De Clercq 2007). In principle, these antiviral drugs are used at concentrations that are not detrimental to the DNA synthesis of the host (Bras et al. 2001; De Clercq 2003; Miller et al. 2005), and are converted to active metabolites by virally encoded thymidylate synthase enzyme. In addition, evidence shows that the SGHV shares a high homology with HHVs, including DNA polymerase and thymidylate synthase enzymes (Kariithi et al. 2013b), which are the antiviral target genes, inhibiting viral DNA replication. Thus, these drugs are ideal chemotherapeutic agents against viral infections in mass-reared insects. Overall, the administration of antivirals results in

reduced viral titre. Reduction of viral shedding results in reduced horizontal transmission of the virus into blood meals during *in vitro* membrane feeding, thereby keeping the virus undetected in the colonies. Success was achieved in controlling the SGHV in the tsetse mass-production facility in Ethiopia and in the Seibersdorf Laboratory; oral administration of valacyclovir (at a dose of 300 µg/mL blood) for over 18 months significantly reduced SGHV loads and suppressed SGH outbreaks to undetectable levels (Abd-Alla et al. 2008) (Fig. 3). Importantly, such long-term administration of valacyclovir resulted in acceptable levels of fly productivity and mortality. It is important to note that valacyclovir reduced viral DNA replication. It will be more effective when it is administered to flies with a low virus titre, but not to flies with a symptomatic infection.

Figure 3. Effects of long-term treatment with the antiviral drug valacyclovir on *G. pallidipes* biology and virus prevalence. SGHV virus loads and SGH prevalence determined by qPCR and dissection, at 60 days after feeding. (---): threshold virus load correlated with SGH symptoms. Negative control: flies fed exclusively on clean blood meals; positive control: flies fed on virus-contaminated blood meals; valacyclovir: flies fed on virus-contaminated blood supplemented with 300 µg/mL valacyclovir. Data points for virus load are for individual flies (Abd-Alla et al. 2012).

3.1.2. Clean Feeding System

In tsetse mass-rearing facilities, the *in vitro* membrane feeding system, which involves feeding of up to 10 sets of fly feeding cages on the same membrane in succession, favours horizontal transmission of the virus (Feldmann 1994; Abd-Alla et al. 2012). A single SGHV-infected individual is able to shed up to 10^7 infectious

viral particles into the blood meal during a 10–15 min feeding event (Abd-Alla et al. 2010) (Fig. 2), thereby infecting most of the non-infected flies in the cages that feed afterwards. This results in the spread and long-term maintenance of the virus in the colonies. Consequently, it was thought that, in principle, the horizontal SGHV transmission can be efficiently interrupted by modifying the membrane feeding regime. This was the origin of the clean feeding system (CFS), which is now one of the most cost-effective anti-SGHV management strategies in tsetse mass-rearing facilities (Abd-Alla et al. 2010, 2013b).

Implementation of the CFS involves providing each fly cage with fresh blood at each meal to prevent flies from picking up the virus shed during the feeding of flies in earlier cages without increasing the cost. The CFS consists of three basic steps: (1) teneral flies and their subsequent progenies are always fed first, (2) after feeding the maximum possible numbers of cages on this first round (determined by the available resources, i.e. number of feeding trays), the rest of the fly cages are fed on the same membranes in a second round, and (3) after the first and second feeding rounds, the remaining colony flies, i.e. the oldest, are fed last on the same membranes (Abd-Alla et al. 2013b). In the CFS setting (Fig. 4), one of the most important issues is to keep separate all the fly records and pupae collection and incubations from these three colony feeding groups. Within two years of its implementation, the CFS was hailed as a big success; virus prevalence was significantly reduced and the SGH symptoms were completely eliminated from the colonies (Abd-Alla et al. 2013b). As a result, no SGH outbreaks have been reported in the colony in the two years since the CFS was put in place at the Seibersdorf Laboratory. It is worth noting that the CFS was implemented using existing resources without adding extra cost.

3.1.3. Combined Approaches

To manage SGHV infections effectively in tsetse mass-rearing, a multi-tactical approach is the best option. Based on experience in dealing with SGHV infections gained over the last decade, some of the extremely important tactics to manage viral infections include: (1) strict sanitation of facilities, equipment and adult cages, (2) regular monitoring of SGH symptoms (fly dissection and PCR diagnosis), and (3) quarantine, e.g. by keeping a virus-free stock colony separate from the main colony. In addition to existing colonies, many new tsetse mass-rearing colonies are being established from field-collected flies in different African regions, with little or no awareness of SGHV prevalence. Since the SGHV is present in wild tsetse populations, there is a risk of introducing the virus to the new colonies.

The implementation of the CFS or administration of antiviral drugs alone in the management of SGHV infections requires a long time to significantly bring down the SGH prevalence to acceptable levels (below 10%). Importantly, the trials described by Abd-Alla et al. (2013b) were based on small fly groups with relatively low SGH prevalence (compared with the usual high prevalence in normal colonies). The CFS or valacyclovir alone reduced the prevalence of symptomatic infections (SGH) to an average of 1.9% and 0.63% within 28 and 21 months, respectively (Abd-Alla et al. 2012). As administration of the antiviral drugs in mass-rearing facilities is very feasible given that these drugs are cheap and readily available

commercially, a combination of the CFS (consisting of sanitation, modified blood feeding, SGH monitoring and quarantine) and the administration of valacyclovir was attempted on a large scale (Abd-Alla et al. 2014). Combined, the CFS and the supplementation of blood meals with valacyclovir resulted in complete elimination of the disease symptoms (from an initial 24% SGH prevalence) within six months of implementation. Taken together, an integrated approach can be used by combining these anti-SGHV strategies.

Figure 4. Handling, feeding and management of a tsetse colony. (A) Regular in vitro membrane feeding system as routinely practised in tsetse mass-production factories, (B) intermediate phase, and (C) final phase of the clean feeding system. WU = weekly unit, WPC = weekly pupal collection (Abd-Alla et al. 2013b).

3.1.4. Impact of Implementing the Combined Approach on Colony Performance

The long-term application (more than 3 years) of antiviral drugs to manage SGHV infections in the mass-rearing of *G. pallidipes* colonies has not resulted in any observable negative effect on fly productivity and survival. The single-most important aspect of the combined approach, in the case of an SGH outbreak, is the ability to restore colony productivity rapidly (within six months) without any

additional cost in specialized equipment or reagents, except that minimal training of staff on colony handling protocols is required. Importantly, in case supplementing blood meals with a drug results in an undesirable side effect, or there is a potential risk of resistance development, the drug can be withdrawn once the SGH outbreak has been successfully managed; thereafter, the CFS protocols alone should reduce an SGHV infection to an acceptable level. Ideally, if frequent and fluctuating SGH outbreaks occur in a mass-rearing facility, the administration of antiviral drugs would reduce SGH prevalence significantly; then the sustained use of the CFS should follow. For rearing facilities with low SGH or non-observable SGH outbreaks, the CFS should be maintained so that that disease symptoms are not manifested.

3.1.5. Novel Prospective Approaches to Manage SGHV in Tsetse Mass-Rearing

Paratransgenesis, i.e. the expression of antiviral molecules by transformed endosymbionts, is a viable option to manage viral infections in tsetse mass-rearing. Paratransgenesis was first achieved in *Rhodnius prolixus* Stål, vector of Chagas' disease, via transformation of its symbiont *Rhodococcus rhodii* (Abd-Alla et al. 2014). This approach has been hailed as a promising strategy in the creation of pathogen-refractory mosquitoes (Beard et al. 1992). *Wolbachia* is a potential endosymbiont for creating SGHV-refractory tsetse strains; it induces antiviral activity in insects (Wilke and Marrelli 2015). Another symbiont that can be targeted for paratransgenesis in tsetse flies is *Wigglesworthia*, which upregulates the host's cellular defence system (Saridaki and Bourtzis 2010; Rances et al. 2012; Weiss et al. 2012). Recently, Boucias et al. (2013) showed that removal of *Wigglesworthia* from *G. pallidipes* significantly reduced SGHV replication in adult flies and suppressed virus vertical transmission to fly progeny. The symbionts can be artificially cultured, genetically engineered and stably fixed into the tsetse genome to express molecules that interrupt SGHV replication and transmission. Such virus-refractory tsetse lines are stable because the endosymbionts are maternally transmitted to the next fly generation. Attempts have been made to identify potential candidate transgenes that can be exploited in creating paratransgenic tsetse lines (Boucias et al. 2013).

Another potential approach to managing virus infections is applying RNA interference (RNAi), which can also be mediated via bacterial endosymbionts (Abd-Alla et al. 2016; Kariithi et al. 2016). The feasibility of this approach is underscored by the presence in the tsetse genome of the key components of the RNAi machinery, including several copies of Dicer (*dcr*), Argonaute (*ago-1* and *2*) and some components of the dsRNA-binding genes (Whitten et al. 2016). On the other hand, the genome of the SGHV contains some of the key genes involved in viral DNA replication, including late effector factors (*lef*), DNA polymerase (*dnapol*), DNA helicase (*dnahel*) and the *per os* infectivity factor (*pif*) genes (IGGI 2014). The identification of these key genes implies that the RNAi system is not only functional in tsetse flies but may also be actively involved in keeping the SGHV under control; apparently only a few of the viral genes are expressed during asymptomatic infections to avoid detection by the host's immune system. Preliminary results

indicate that RNAi-mediated silencing of SGHV genes is feasible (I. K. Meki, personal communication).

3.1.6. Technology Transfer from the Laboratory to Large-Scale Mass-Production Facilities

It is a challenge to transfer the technology of a newly developed pathogen management strategy from small-scale laboratory trials to a wider operational application. This is particularly so because, unlike controlled laboratory settings, external uncontrolled environmental factors could limit the technology's effectiveness under mass rearing operations. Even before the technology is transferred to operational applications (in mass-rearing, and in the field where applicable), there is the challenge of sustaining the technology in the laboratory. For example, in the case of the application of transgenic and RNAi approaches, the stability of the transgenes requires finding ways to maintain the transgene constructs (preferably as embryos) and to make rigorous evaluations under mass-rearing conditions. These embryonic transgenes can be used as stock for the operational applications. If there is no effective means of ensuring the stability of these technologies under mass-rearing conditions, the advantage of having a fall-back plan is jeopardized because the transgenes will be lost. So far, most laboratory trials have not been successfully transferred to the operational level. However, there is a noteworthy exception -- the IAPV-RNAi laboratory trial was field-tested in beehives in the USA (Abd-Alla et al. 2016; Kariithi et al. 2016). Also, transgenesis in silkworm mass-rearing has made significant progress in technology transfer from the laboratory to contained trials at operational level in multiple locations (Hunter et al. 2010).

The case of the combined anti-SGHV management strategy developed at the IPCL tsetse production facility in Seibersdorf is an example of how technology can be successfully transferred from laboratory trials to an operational level with widespread field application (FAO/IAEA 2015a). The technology was transferred to a tsetse mass-rearing facility in Kality, Ethiopia. This was done primarily by developing standard operational procedures (SOPs), training staff in the mass-rearing facility on the correct manner of implementing the new technology and establishing ways to evaluate it. The implementation of the management package resulted in the complete elimination of SGH symptoms and, moreover, according to PCR, eliminating the GpSGHV infection (Fig. 5).

3.2. Management of CpGV in Mass-Rearing the Codling Moth

In addition to infection with pathogenic fungi, microsporidia, nematodes and bacteria, mass-reared colonies of the codling moth are contaminated with *C. pomonella* granulovirus (CpGV) (Subbaiah et al. 2013). CpGV infections are mainly observed in larvae, but the infections are largely asymptomatic. CpGV is an extremely specific and highly virulent viral pathogen of the codling moth. Once ingested by the larvae, the virus infects the digestive tract causing the disease which kills within 3–7 days. The subsequent rupturing of the larval skin allows the CpGV virion particles to infect other larvae and to spread in the colony. The routes of entry

of CpGV into mass-rearing facilities are through the air filtration systems, walls of the rearing rooms and spent diet (Zimmermann et al. 2013). Since handling is the main contributor to accidental contamination of the codling moth with CpGV (Cossentine et al. 2005), the most common practices to control CpGV infection include adding formaldehyde to the larval diet (Dyck 2010; Zimmermann et al. 2013) and general hygiene, e.g. washing egg sheets and disposing of spent diet and dead larvae.

Figure 5. Impact of implementing a combined virus management strategy on SGH prevalence in *G. pallidipes* in the tsetse mass-rearing facility in Kaliti, Ethiopia.

An alternative strategy to control CpGV infections is to establish virus-free laboratory colonies. This can be achieved by using codling moth strains that are known to be CpGV-resistant. CpGV-resistant strains have previously been reported in various commercial orchards in Europe routinely subjected to CpGV treatments (Schmitt et al. 2013). The fact that the CpGV is vertically transmitted (transovum) in codling moth colonies (Brinton et al. 1969) can be exploited in the establishment of virus-free colonies. For example, surface decontamination of the eggs sheets can successfully remove low levels of CpGV infection. This is a cheap procedure -- a 20-min egg sheet swabbing in simple solutions such as 0.5% sodium hypochlorite/1% Tween 20, or a 6-h UV-B exposure, followed by rinsing with distilled water to maintain egg viability (Etsel and Falcon 1976; Dyck 2010). Once such a virus-free colony is established, the possibility that a low-level virus infection still remains can be monitored easily by PCR. Primer and PCR amplification protocols for the detection of CpGV in codling moth populations have been described (Kundu et al. 2003).

3.3. RNAi Approach to Manage Pathogens of the Honeybee

In the absence of effective antiviral chemotherapeutics in hives of the European honeybee (*Apis mellifera* L.), symptomatic viral infections are minimized by reducing stress, e.g. maintaining healthy colonies, and chemical control of *Varroa*, disinfection and selection for disease-resistant honeybee races (Chen 2011; Martin et al. 2012). Modern antiviral strategies include RNA interference (RNAi), which can significantly reduce viral titres up to 100% and mortality up to 90%. Notable success stories include the reduction of honeybee mortality caused by the Israeli acute paralysis virus (Maori et al. 2009), significant reduction of infection (27–60%) of the Asian honeybee *Apis cerana* F. with the Chinese sacbrood virus (Liu et al. 2010) and protection of European honeybees against infection with the deformed wing virus (Desai et al. 2012). Additionally, the threat caused by *Varroa* mites (viral reservoirs, incubators, activators and competent viral vectors) can be significantly reduced, up to 97%, by RNAi (Campbell et al. 2010; Garbian et al. 2012).

3.4. Transgenic Approaches to Manage Pathogens of the Silkworm

There are no curative measures against viral diseases in silkworms. Conventional management measures include preventing virus transmissions (destroying the virus transmission cycle and breeding for resistant races), enhancing silkworm vigour (rearing hardy races and eliminating unfavourable conditions), diagnosing early viral disease (culling out sick worms and therapeutics), and taking prophylactic measures (disinfecting silkworms and rearing sheets). These methods are inexpensive and quite effective, but are not sufficient. Modern (transgenic) antiviral methods have been successfully applied in the silkworm industry without compromising the cocoon crop yields, resulting in enhanced resistance to baculovirus (*Bombyx mori* nucleopolyhedrovirus BmNPV) in silkworms (Watanabe 2002; Isobe et al. 2004; Kanginakudru et al. 2007; Jiang et al. 2012a, b; Subbaiah et al. 2013; Jin et al. 2014). The effectiveness of the transgenic silkworms could be further optimized by combinations of various molecular technologies. The best approach would be pre-selecting silkworm parents with a substantial level of natural virus-resistance, creating hybrids and enhancing their refractoriness to virus infection.

3.5. Management of Non-Viral Diseases in Insect Mass-Rearing

Among the non-viral disease-causing agents in mass-reared colonies are: bacteria (Bacillaceae, Pseudomonadaceae and Enterobacteriaceae), rickettsia (Rickettsiaceae), protozoa (Amoebias, Gregarine and Coccidian), fungi (Entomophthorales and Muscardine), microsporidia and spiroplasma (Jiang et al. 2013a, b). Most of these contaminating microbes are acquired through the diets, and can alter the nutritional value of insect diets. Deliberate measures must be put in place to monitor microbes, e.g. using bioassays, and then prevent microbial contaminations because they are a major problem affecting the mass-rearing of insects (Sikorowski and Lawrence 1994).

Most of the bacterial diseases affect the larval stages of the insects (Gouli et al. 2011). Although the majority of these entomopathogenic bacteria are transmitted orally, they can also be vectored by nematodes and parasitoids. On the other hand, fungal diseases are transmitted (via infectious asexual spores) through penetration of the insect cuticle, but a few are orally transmitted. Upon infection, the fungus grows in the hemolymph and produces lethal toxins. New spores can be produced after the death of the host, thereby spreading the fungal infection in the colony. Amongst the bacterial control measures are disinfection and pasteurization, e.g. combinations of heating and antimicrobials to inhibit microbial growth. This approach should be practiced in combination with decontamination of the diet and rearing containers. For example, in tsetse mass-rearing, the feeding trays and membranes are frequently autoclaved, while the blood diet batches (which are usually decontaminated by irradiation prior to delivery to the mass-rearing facilities) are routinely monitored with petri dishes for contamination. In addition, other control measures include strict observance of personal hygiene of the workers, maintenance of a clean and sanitary environment, and sterilization of insectary equipment. Inflow and accumulation of air-borne microbial contaminants can be effectively prevented by air filtration (Feldmann 1994; Sikorowski and Lawrence 1994; FAO/IAEA 2006, 2015b, 2019).

Microsporidia are close relatives of fungi, and are transmitted either orally (via spores) or vertically (via eggs) (Lietze et al. 2010). Upon infection, these pathogens develop within the cytoplasm, eventually causing death, either directly (due to cellular damages) or through septicaemia (via secondary bacterial infections of the damaged cells). The highly species-specific microsporidia often infect insect populations chronically without disease epidemics, but they can be fatal in some mass-rearing facilities (Eilenberg et al. 2015). Microsporidia can be controlled by biennial (autumn and spring) applications of antibiotics, e.g. Fumagillin in honeybee colonies, but some species have shown signs of resistance to antibiotics (Huang et al. 2013). Applying antibiotics is usually combined with replacing old, dark brood combs.

Entomopathogenic rickettsia and spiroplasma are efficiently transmitted vertically and horizontally; a high prevalence of these pathogens results in mortality and severe losses of fecundity and general colony fitness. Due to the nature of their transmission in mass-production settings, i.e. mainly through contact, various infrastructural improvements can be implemented to significantly manage their infections and to improve rearing quality. These include circulating water and air filtration systems, continuous monitoring and waste removal, continuous larval/pupal separation and sterilization (routine hygiene), appropriate measures to reduce and prevent the development of pathogens, and a pathogen-free optimal diet.

Artificial mass-rearing conditions eventually lead to a decrease in the immune fitness of the insects (Yates and Antia 2006; Hawley and Altizer 2011; Ugelvig and Cremer 2012). Therefore, to replenish colonies with individuals having a high immune capacity, it is necessary to replace periodically or outbreed the colonies with fresh pathogen-free field-collected samples/specimens. For example, the colonies could be outbred with locally caught conspecifics (which should be PCR-screened for pathogen infection before introducing them into existing colonies). It is strongly recommended that, in the case of mass-rearing tsetse flies, newly

introduced conspecifics should be maintained under the above-mentioned combined approach to prevent an SGH outbreak.

4. CONCLUSIONS

Pathogens affect the quality of mass-reared insects. Therefore, it is very important to manage microbial and in particular virus infections in insect colonies during the mass-rearing process. Although there is still much to be learned about insect pathogens, in general microbial and fungal pathogens are currently managed using antibiotic and anti-fungal compounds, in addition to implementing the required sanitary procedures, but viral disease management when mass-rearing moths requires the use of formalin. However, for working staff, formalin is a hazardous chemical, and also it cannot be used to manage viral diseases when mass-rearing tsetse flies or mosquitoes. Therefore, alternative virus management strategies are needed, and thus this chapter focused on the management of viral pathogens. Inglis and Sikorowski (2009a, b) reviewed in detail the management of other insect pathogens.

Applications of transgenics and RNAi-gene silencing drugs, and the oral administration of antiviral drugs, have proven to be potent measures in managing viral infections in tsetse mass-rearing, apiculture and sericulture. However, it is clear that no single method can be used alone to manage effectively viral infections in insect mass-rearing; instead, an integrated approach is required. Molecular-based methods are a good complement to conventional virus management practices. The application of these measures should be accompanied by accurate and routine diagnosis of infections, and proper documentation of disease manifestations. To avoid the risk of developing drug-resistance, the application of antiviral drugs, especially in tsetse mass-rearing, should be restricted to colonies with comparatively high SGH outbreaks; the unnecessary administration of antiviral drugs to healthy colonies should be avoided. For colonies with a low SGH prevalence, a best management practices (BMP) approach entails applying preventive measures such as CFS and an avoidance of stressors as the main pillars, while not using antiviral drugs. To develop sustainable virus management strategies for mass-rearing insects, the link between covert and overt infections needs further investigation.

There are several important aspects to note in managing pathogens: (1) it is important to monitor the occurrence of disease symptoms and perform regular diagnostics, (2) a hygienic and optimal environment in the colonies must be maintained; all insects showing disease symptoms must be removed immediately and appropriately destroyed, (3) insect colonies should be kept in different stocks – keeping them separate from the mass-rearing is easier than managing an outbreak that infects the whole facility, (4) it is advisable to outbreed colonies at intervals (to replenish genetic diversity, if possible with strains that are pathogen-resistant), and (5) hybrid lines should be reared separately; these can be used to establish highly productive colonies in the event that the productivity of the parent colony declines.

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