Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

Entomology Unit

Activities Report

2009

FAO/IAEA Agriculture & Biotechnology Laboratory
IAEA Laboratories Seibersdorf
International Atomic Energy Agency
Vienna, Austria

The picture on the front cover shows 4 members of the *Bactrocera dorsalis* complex, including major horticultural pests of Asia/Pacific origin, that have been also invading Africa and the Indian Ocean region. Top left: *Bactrocera dorsalis*, top right: *Bactrocera philippinensis*, bottom left: *Bactrocera papayae*, and bottom right: *Bactrocera invadens*

*A new tsetse colony (*Glossina palpalis gambiensis*) was established in 2009 by the tsetse research group with seed material from the colony maintained at the Centre International de Recherche-Développement Sur l'Elevage en Zone Subhumide (CIRDES), Bobo Dioulasso, Burkina Faso. The colony was established to carry out radiation studies and other work in support of the tsetse control programme in Senegal. Irradiation of 25, 27 and 29 day-old pupae with 110 Gy resulted in high quality males that were almost completely sterile. An evaluation has been initiated to assess the effects of chilling and irradiation on male adult sterility, performance and behaviour. Initial tests showed that male pupae (after the female emergence flush) could be chilled at 10 and 12.5 ºC for 7 days for transport to the target locations without fly emergence. In field cage tests, males that were chilled as pupae for 7 days at 10 ºC competed slightly less well for receptive females than un-chilled males.*

Good progress has been made with the work on the virus of Glossina pallidipes *and in understanding the various aspects determining the dynamics of the virus in the colony. Flies, which were found to be negative or slightly infected with the virus using a non-destructive PCR, were asymptomatic (no symptoms of salivary gland hypertrophy (SHG)), whereas 85% of the highly PCR positive flies showed SGH. Flies with symptoms of SGH released much more virus particles into the blood during feeding (10 million particles) as compared to asymptomatic infected flies (only 100). Increasing the number of infected blood feeds given to PCR negative flies increased the proportion of flies secreting virus into the blood. Feeding flies each time with fresh blood (i.e. a clean feeding strategy), rather than feeding several cages of flies on the same membrane and blood reduced the number of virus particles in the flies significantly after 2 generations. Feeding flies with the antiviral drug Valacyclovir reduced the productivity of the female flies after 3 generations, whereas productivity remained acceptable with Acyclovir. Flies that received the antiviral drugs did not show symptoms of SGH. The work on RNAi treatments and the use of specific antibodies continues, but the data so far are not conclusive and further work is needed.*

Dose mapping of the X ray machine (RS2400^{TM}) was completed, showing a good dose uniformity ratio of 1.3:1, which is very suitable for the purpose of insect sterilization.

The fruit fly rearing and quality management group, in support of a new Coordinated Research Project with the participation of 20 countries, carried out a series of hybridization tests in the laboratory with members of the Bactrocera dorsalis *pest complex. Crosses with* B. dorsalis*,* B. invadens*,* B. philippinensis *and* B. carambolae *were all successful in that interspecific matings occurred and that the hybrid offspring was viable. These interspecific matings were confirmed under semi-field conditions with trees in field cage tests. Work was*

also carried out to characterize these different species using morphometric measurements of eggs and pupae in relation to weight.

Significant progress was made with the development of rearing methods for the olive fly, i.e. (1) the use of waxed oviposition panels increased the oviposition by olive fly females, and (2) a male:female sex ratio of 2:1 resulted in the highest production of fertile eggs.

A genetic, cytological and molecular analysis was made of two genetic sexing strains of Mediterranean fruit flies, the first maintained at the El Pino Facility in Guatemala and the second one maintained in the CDFA facility in Hawaii. Neither of the two strains showed the expected characteristics and both strains showed components characteristic for VIENNA 7 and VIENNA 8. The strain from Guatemala showed all the features of VIENNA 8, except the inversion, and the Hawaii strains showed a mixture of mtDNA haplotypes. Continuing support is being provided to FAO and IAEA Member States with the screening of fruit fly strains, supply of seed material and the development of genetic sexing strains (e.g. Bactrocera cucurbitae *from Mauritius).*

Progress was likewise made in the development of rearing methods for Anopheles *mosquitoes. A new larval holding tray was developed and is ready for larger scale testing. A new rack system that can hold 50 of the larval trays was designed and a first prototype constructed. The larval-pupal separator was refined and showed great promise using cold temperatures and a vortex to exploit the differences in buoyancy of larvae and pupae. A new blood feeding system consisting of a Memotek heating plate and a membrane to hold the blood was likewise developed and showed promising results. A new diet composed of bovine liver powder, tuna meal and a vitamin mixture was developed and will replace the Koi Fish Food diet. A new flight tube system was tested that could be used as a quality control tool. Further work with* Anopheles arabiensis *showed that males mature sooner (after 11 h after emergence) than previously expected (24 h.), which has implications for experimental work and radiation studies. Several of these new developed mass-rearing techniques for* Anopheles *mosquitoes are being transferred to Sudan and La Réunion for further validation under field conditions.*

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1. PROGRAMMATIC AND UNIT OBJECTIVES

The vision and goals of the Insect Pest Control sub-programme are to increase food security, reduce food losses and insecticide use, and overcome constraints to sustainable rural development and agricultural trade through the development and application of the Sterile Insect Technique and related biological methods in support of area-wide integrated pest management (AW-IPM) programmes to control key insect pests of agriculture and human health. The sub-programme achieves these goals through:

1) Improving procedures and capacities for risk assessment and management of major trade related insects pests of crops through the integration of the Sterile Insect Technique in control and eradication programmes

2) Developing the Sterile Insect Technique and other nuclear based biological control methods to manage risks to agriculture and the environment from exotic insect plant pests

3) Strengthening expertise and capacities in Member States to integrate the Sterile Insect Technique in area-wide integrated pest management approaches against tsetse and screwworm populations

4) Developing and transferring technology and improving capacity building in Member States for the use of the Sterile Insect Technique for the control of malaria transmitting mosquitoes

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3. RESEARCH AND DEVELOPMENT ACTIVITIES

3.1. Tsetse Research Group

3.1.1. Tsetse Colony Status

3.1.1.1. Changes to the Seibersdorf colonies

The Insect Pest Control Laboratory has continued to adjust the tsetse colony holding to address the requirements of the Insect Pest Control Sub-programme.

The most important change this year has been the establishment of a *Glossina palpalis gambiensis* BKF colony from the colony maintained at the Centre International de Recherche-Développement Sur l'Elevage en Zone Subhumide (CIRDES), Bobo Dioulasso, Burkina Faso. We received a total of 8 000 pupae in four shipments in January and February 2009. The colony quickly built up to 10 000 females and was stabilized around this level (**Figure 1**).

In addition to the CIRDES material we also received pupae from the field in Western Senegal to establish a colony (see next section).

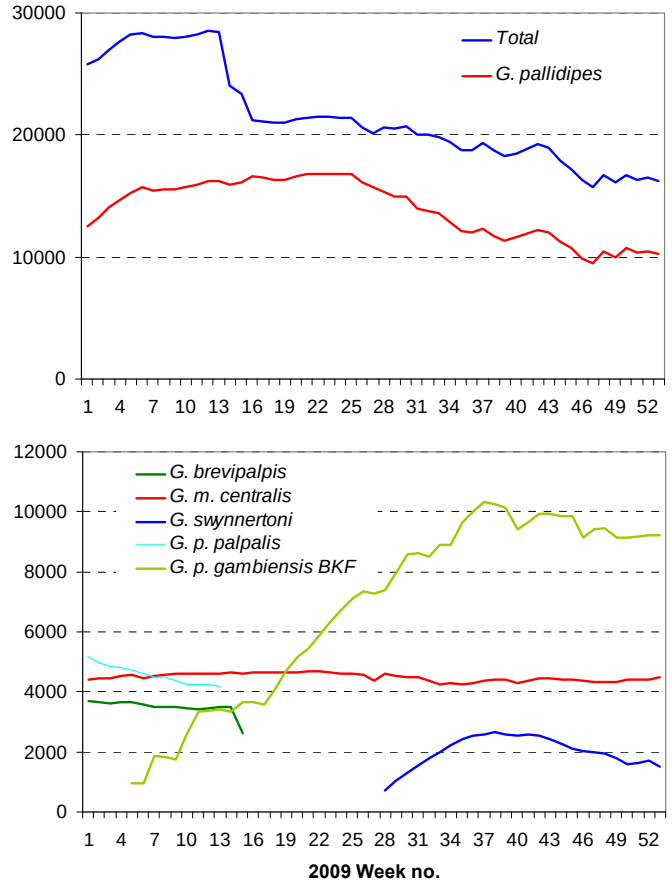

Figure 1. Changes in the Seibersdorf tsetse colonies during 2009.

The number and complexity of the experiments on *Glossina pallidipes*, particularly in relation to the Salivary Gland Hypertrophy Virus (SGHV) (see below) together with the new *G. p gambiensis* BKF colony would have put too large a load on the technical staff. We had, therefore, to reduce/eliminate other colonies to decrease the routine colony holding work. The *Glossina palpalis palpalis* colony was given to the Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Montpellier, France and the *Glossina brevipalpis* colony was given to Onderstepoort Veterinary Institute (OVI), South Africa to supplement their existing colony. Both these colonies were terminated in the early part of the year. The *G. pallidipes* colony also declined from 15 000 to 10 000 due to the shipping of a significant number of pupae to Ethiopia.

Apart from the main colonies we have maintained two small colonies of *Glossina swynnertoni* and *G. brevipalpis* red eye mutant. The *G. swynnertoni* colony has been held in the Insect Pest Control Laboratory for many years. It did not grow due to low fecundity and variable mortality, but improved fecundity over the past year has lead to improved growth and it reached about 2500 females late in the year.

During 2009 we also established a "clean fed" *G. pallidipes* colony (see below). This colony, where each fly received clean blood with each feeding and is kept for experimental work, reached 1500 by the end of the year.

More than 101 000 pupae were shipped during 2009 to nine collaborating institutions.

3.1.1.2. Establishment of a colony of Glossina palpalis gambiensis *from Senegal*

The establishment of *G. p. gambiensis* colony from Senegal in the Insect Pest Control Laboratory was started in mid October 2009 with the receipt of the first 14 pupae produced from wild females collected in the project target area and maintained in a temporary insectary in Dakar. The second batch sent by airfreight was received on 23 November 2009. It was composed of 60 pupae of the first *G. p. gambiensis* strain collected in the area of Pout and 8 pupae produced by the second strain collected in the "Parc National de Hann" in Dakar.

The preliminary data are summarized in **Table 1.** In total, 30 female and 36 male flies have emerged from Pout pupae and 3 females and 4 males from Hann pupae. The related emergence rates were 92.9% and 88.3% for the two pupae batches from Pout and 87.5% for Hann.

Table 1. Emergence, sex ratio and pupal weight of *Glossina palpalis gambiensis* SEN pupae

	Pupae batch	Mean pupal weight (mg)	Emergence			Sex- ratio	
			Females	Males	%	Females	Males
***G. p. gambiensis* SEN-Pout**	14	18.48	7	6	92.86	53.85	46.15
	60	21.01	23	30	88.33	43.40	56.60
***G. p. gambiensis* SEN-Hann**	8	19.89	3	4	87.5	42.86	57,14

The flies were kept in individual holding tubes and offered blood meals on a daily basis. The feeding response of these newly introduced flies varied between nil and 33% with an average of 11.9% on their first day of emergence. On the following days the blood uptake rate reached about 60%. Mating duration recorded from pairs kept in individual tubes gave an average of 1 hour 7 minutes for the first time and about 17 minutes for re-mating. On 31 December 2009, the *G. p. gambiensis* SEN-Pout colony totaled 16 females and 4 pupae. For Hann, 2 of the 3 emerged females were alive. The holding/ handling protocol was slightly modified to ensure a better survival of the flies.

3.1.2. Chilling and Irradiation of Glossina palpalis gambiensis *Pupae*

The assistance of the Agency has been requested by the Government of Senegal to determine the optimal conditions for irradiation, packaging and transportation of male *G. p. gambiensis* pupae to ensure good quality and competitiveness of emergent sterile males, in support to an ongoing tsetse eradication project in the Niayes. The required applied research started, as described above, with the establishment in the Insect Pest Control Laboratory of colonies of two strains of the target species, *Gpg* BKF originating from reared flies from CIRDES of Bobo-Dioulasso, Burkina Faso and from wild flies from Senegal (*Gpg* SEN).

3.1.2.1. Radiation sensitivity to gamma rays of Glossina palpalis gambiensis *BKF in late pupal stage*

Batches of pupae were gamma irradiated on day 25 and day 27 post larviposition (PL) at different doses between 40 and 120 Gy. These days were chosen to allow for sufficient time for shipment to the target area. Preliminary results showed no significant differences between irradiating on the 25th or 27th day PL. A subtotal sterility of at least 96.4% is induced in colony females mated with male flies irradiated as pupae between 110 and 120 Gy.

Due to the long pupal incubation period of 32-34 days to first female emergence at 24°C observed with the BKF strain, additional tests were performed by irradiating batches of pupae on day 29 PL (**Figure 2**). A similar index of sterility was recorded. These results will be useful in combination with pupal scanning with the SK Near Infrared Spectroscopy for pupal sex separation in this species. Irradiation at the pupal stage, specifically on days 25, 27 and 29 PL, does not affect the emergence rate which varied from 94 to 97%.

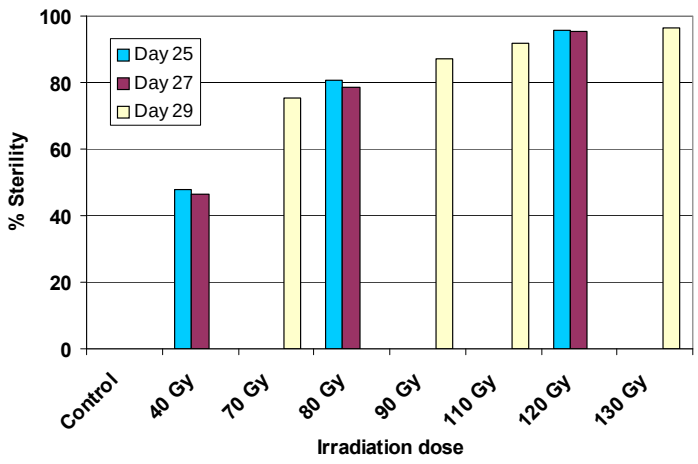

Figure 2. Sterility induced by gamma irradiation of *Glossina palpalis gambiensis* BKF pupae at day 25, 27 or 29 post larviposition at selected doses.

3.1.2.2. Effects of chilling on male pupae

The effect of cooling on the male pupae remaining after female emergence has also been evaluated. Pupae collected on a daily basis were incubated at 24°C and RH 75%. The female flies were allowed to emerge and the remaining, male pupae were used for the experimental treatments kept under lower temperatures for up to 7 days and one treatment kept as a control under normal incubation conditions. Chilling at 10 and 15ºC has been completed and a second test using 12.5ºC is being evaluated (**Table 2**).

Table 2. Percentage emergence of *Glossina palpalis gambiensis* BKF male pupae after chilling at selected temperatures for different durations. The pupae were 34 days old at the start of chilling.

Chilling duration (days)	0	3	5	7
10°C	93	93	90	90
15°C	94	92	93	91
12.5°C (*incomplete*)	100	96	96	95

The emergence of male flies was completely inhibited for 7 days by chilling at 10ºC. The preliminary results showed no significant differences in fly performance (emergence rate and male survival following the cooling period) between the control group and the experimental treatments kept at 10ºC during 3, 5 and 7 days (**Figure 3**).

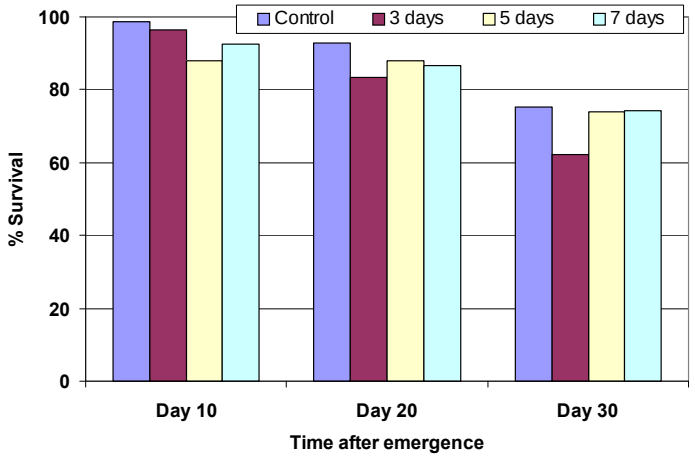

Figure 3. Survival of male *Glossina palpalis gambiensis* BKF at various times after chilling at 10ºC for 0, 3, 5 or 7 days.

During chilling at 15ºC, a total of 60% of the adult males emerged during the 7 days chilling (**Figure 4**) indicating that such a cooling temperature could not be used for the transportation of male pupae from Burkina Faso to Senegal.

Further investigations are being undertaken to have a complete evaluation of the effects of chilling and irradiation on the flies to finalise the determination of the optimal condition for handling, irradiation and transportation of male pupae.

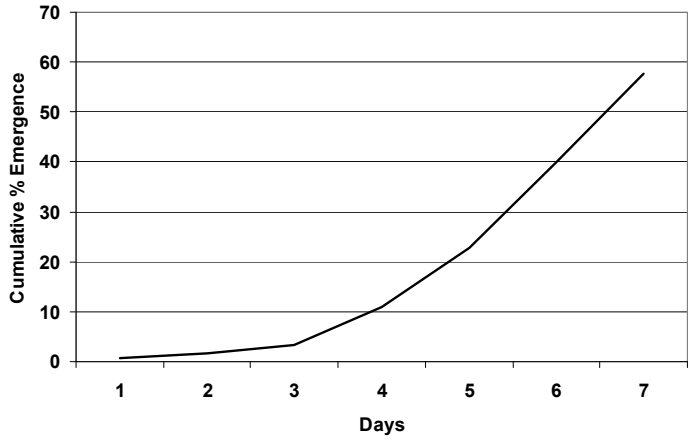

Figure 4. Emergence of male *Glossina palpalis gambiensis* BKF during incubation at 15ºC.

3.1.3. Salivary Gland Hypertrophy Virus

As reported in previous Activity Reports, some tsetse species carry a virus (**Figure 5**) that, in a certain proportion of individuals, leads to salivary gland hypertrophy (SGH) and these individuals also show reproductive abnormalities. In natural populations the prevalence of the virus is low (0.5-5%) based on salivary gland dissection. In a colony of *G. pallidipes* that originated from Uganda and is maintained in the Insect Pest Control Laboratory, the frequency of SGH ranged from 4-10%. However, PCR analysis has confirmed that the virus is present is almost 100% of laboratory colony flies. The virus was also detected in samples of *G. pallidipes* from the colony maintained at the Kaliti facility in Ethiopia. Due to the negative impact of the virus on colony productivity under certain stressful conditions, it is important to understand more about the virus with the goal of developing a strategy for its management. The most effective way to begin this study is to understand more about the virus in terms of its taxonomy through nucleotide sequence information. Beside sequence analysis, the work aimed to gather information about the biology of the virus and its relation to hypertrophy symptoms and sterility. The complete genome sequence was determined and published. The information obtained from the genome sequence provides opportunities for i) the use of PCR and quantitative PCR (qPCR) to analyse the prevalence and dynamics of the virus, ii) to develop RNA interference (RNAi) technology as a potential tool for the development of virus management protocols, iii) produce virus specific antibodies to be used for neutralizing the virus infection. In addition, work on the effect of some antiviral drugs to suppress viral replication, experiments to understand virus transmission in the colony, and the impact of clean feeding on virus prevalence were carried out.

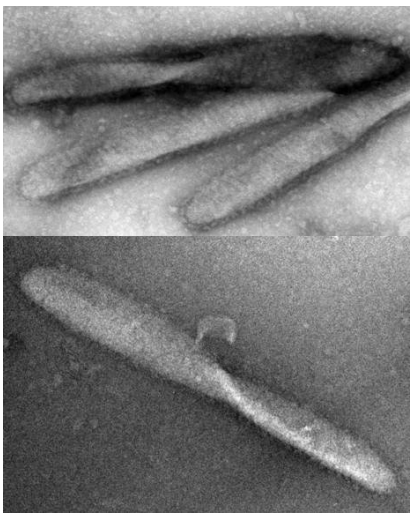

Figure 5. Purified SGHV under Cryo-EM.

3.1.3.1. Hypothesis on virus dynamics in the Glossina pallidipes *colony*

The high prevalence of the SGHV in *G. pallidipes* (GpSGHV) in the Seibersdorf and Kaliti colonies, in comparison to the relatively low prevalence under field conditions, was the subject of many questions. Transmission of the virus is thought to be vertical, from mother to progeny and horizontal, from one fly to another during feeding especially in laboratory colonies where *in vitro* membrane feeding is used. As reported in the 2008 Activity Report, symptomatic infected males are sterile and symptomatic females have a reduced fecundity, but their progeny are all symptomatic and sterile. As a consequence**,** the main expected route for SGH maintenance in the colony is horizontal transmission of the virus from infected flies to non infected flies through blood during feeding (**Figure 6)**. This hypothesis was the subject of several experiments to confirm virus transmission via contaminated blood to infect other flies (see the following section), and also was the basis of the virus management strategies (i.e. using the neutralization of virus infection using virus specific antibodies and using "clean" blood feeding) in order to block horizontal transmission. When non-infected flies acquire a virus infection by feeding on contaminated blood, the acquired virus needs to replicate to increase the number of virus particles and to convert the asymptomatic infection

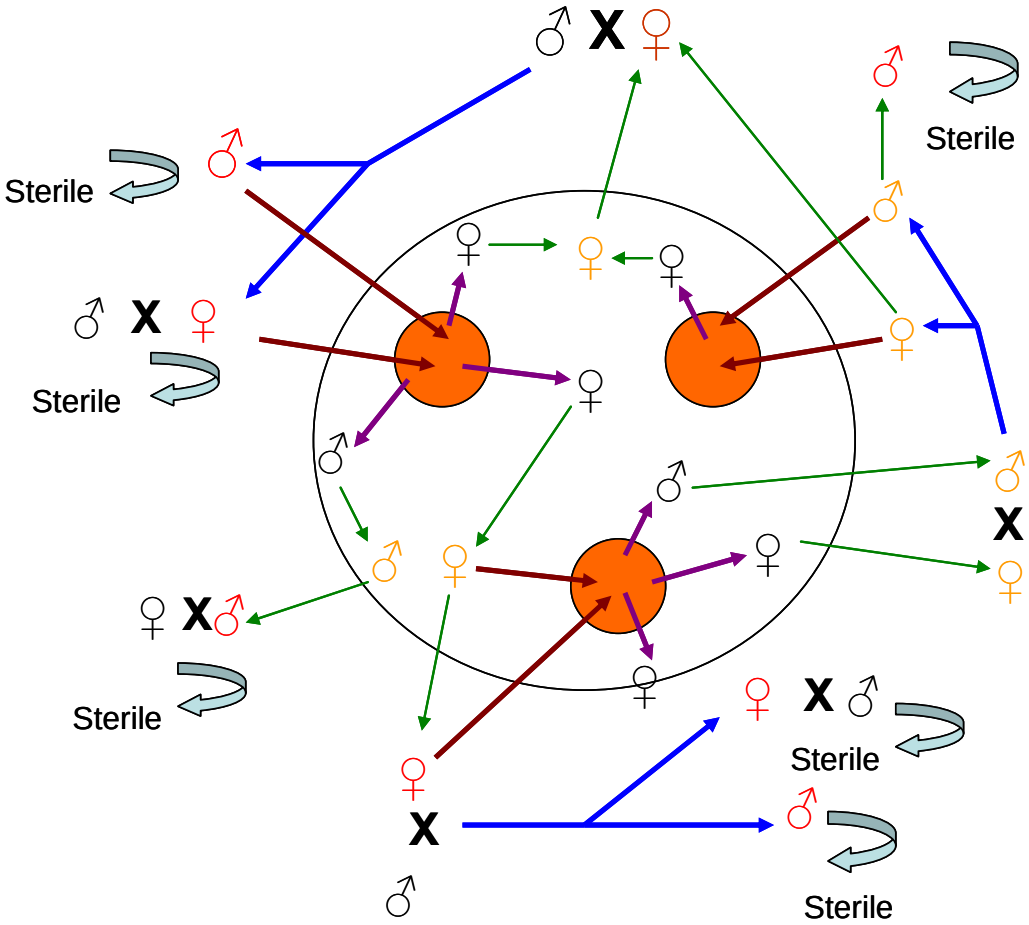

Figure 6. SGHV dynamics in *Glossina pallidipes* colony. Orange circle represent feeding membranes, ♂♀ colours indicate the virus infection state = Black: negative, Orange: slightly positive, Red: highly positive with SGH. Arrow colours: Blue: vertical transmission, Brown: horizontal transmission (positive flies release virus to blood), Violet: horizontal transmission (negative flies take up the virus from contaminated blood), Green: increasing virus inside infected flies by multiplication. Thick and thin arrows indicate confirmed and assumed facts, respectively.

into a symptomatic one. Although this scenario is not yet confirmed in detail, it is most likely to be a factor, as indicated by comparison with other DNA insect viruses and from the observed high virus copy number in symptomatic flies in comparison to asymptomatic infected flies. A commercial antiviral drug and RNAi technology are being tested to block virus replication in infected flies as the basis to manage the virus infection in *G. pallidipes* colonies. Also, asymptomatic infected flies release virus into the blood during feeding and the asymptomatic infected flies can most probably produce some symptomatic infected progeny, particularly if they are challenged with virus in the blood.

3.1.3.2. Detection of virus level in live flies by a non-destructive PCR

To assess the first hypothesis related to horizontal transmission, which involves the release of virus by infected flies into the blood during feeding, and to explore the relationship between the infection status and the quantity of virus particles released, it was necessary to identify the infection status in live flies using a non-destructive PCR. Then flies with identified infection status would be fed on blood and the virus copy number would be determined by qPCR in the blood after feeding. An intermediate leg was excised from teneral flies and the total DNA was extracted and used for endpoint PCR. The PCR products were analysed on 1.5% agarose gel and based on the band density the flies were divided into three groups: i) negative flies, ii) slightly positive flies and iii) highly positive flies **(Figure 7)**. A correlation between the PCR results and fly dissections indicate that while all negative and slightly positive flies were asymptomatic, 85% of highly positive flies showed salivary gland hypertrophy.

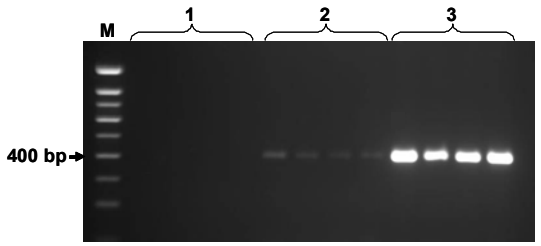

Figure 7. Detection by PCR of SGHV DNA extracted from intermediate excised leg. 1: negative flies; 2: slightly infected flies and 3: highly infected flies; M: SF smart DNA ladder (Eurogentec).

3.1.3.3. Quantification of virus copy released into blood during feeding from symptomatic and asymptomatic flies

Teneral flies with different infection status, selected as described above, were fed individually on 200 µl blood/fly. After feeding, the blood remaining under the membrane was recovered and total DNA was extracted and used for qPCR. The results indicate that flies with SGH injected around 10 million virus particles into blood during feeding, while the asymptomatic infected flies released around 100 virus particles on average (**Figure 8**). This result confirms the first step of virus horizontal transmission and indicates that both symptomatic and asymptomatic infected flies released virus particles into blood during feeding. It confirms also the strong correlation between infection status and the quantity of released virus.

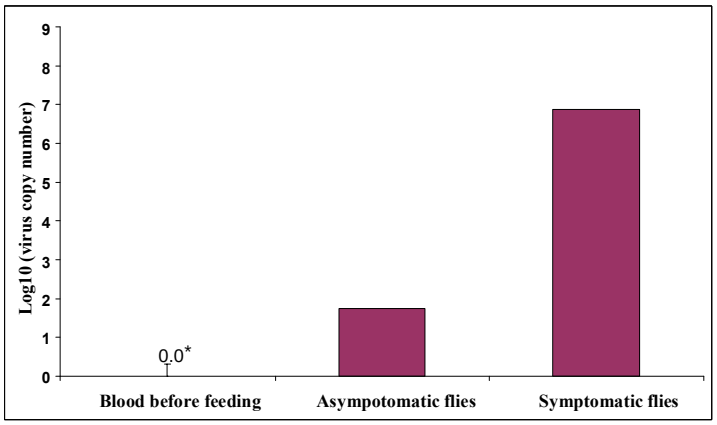

Figure 8. Virus copy number (log_{10}) in blood before and after feeding asymptomatic virus-infected and symptomatic *Glossina pallidipes* (Tororo) colony flies.

3.1.3.4. Impact of feeding virus contaminated blood on virus secretion in the saliva

To confirm the second hypothesis of horizontal virus transmission, PCR negative teneral flies were given 1, 3, 5 or 7 feeds on blood contaminated by feeding on it first infected flies. Following the contaminated feeds the flies were "clean fed" during a total of 7 feeds, and then the blood residue after feeding the 8th blood meal (with clean blood) was analysed for the presence of virus. The results indicate that with increasing numbers of feeds the proportion secreting virus with the saliva into the blood progressively increased **(Figure 9)**. This result clearly confirms that horizontal virus transmission occurred in *G. pallidipes* colonies that use the *in vitro* membrane feeding where multiple feeds are made on the same membrane. Consequently, horizontal transmission could be considered as the main route for virus contamination in these colonies.

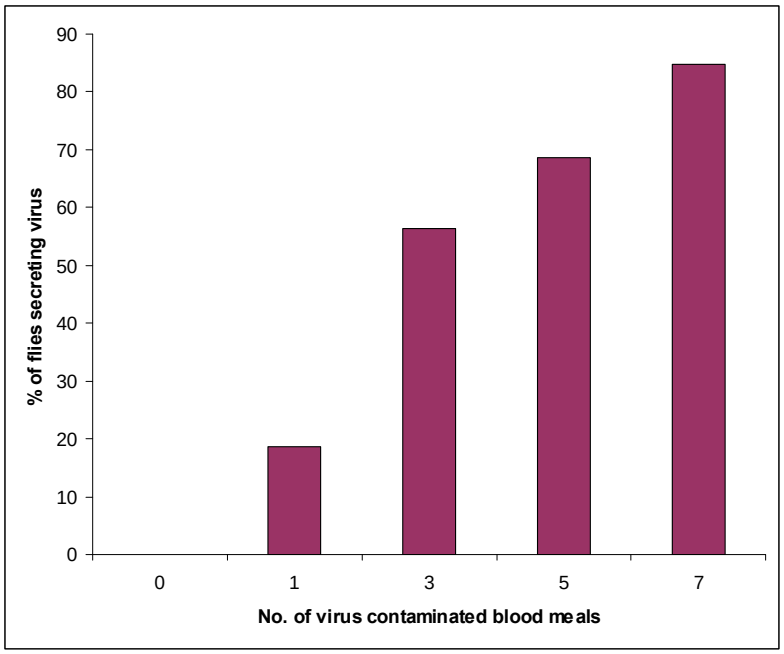

Figure 9. The percentage of flies secreting virus into the blood during feeding in relation to the number of virus-contaminated blood feeds.

3.1.3.5. Impact of feeding clean blood on the virus load in flies

Although it was realized a long time ago that *in vitro* membrane feeding may promote horizontal transmission, feeding multiple batches of tsetse flies fed on the same membrane is economically advantageous. This system has been very successful to mass rear other tsetse species in the absence of salivary gland hypertrophy virus infection. After confirming the importance of virus horizontal transmission we investigated the effect of feeding *G. pallidipes* flies on clean blood, by using a clean membrane and tray for each feed, on the prevalence of the SGHV in a *G. pallidipes* colony. Teneral flies were fed on clean blood for three successive generations and the virus load was estimated in each generation by qPCR. The results indicated a significant decrease in the virus copy number after three generations **(Figure 10)**. While around 10

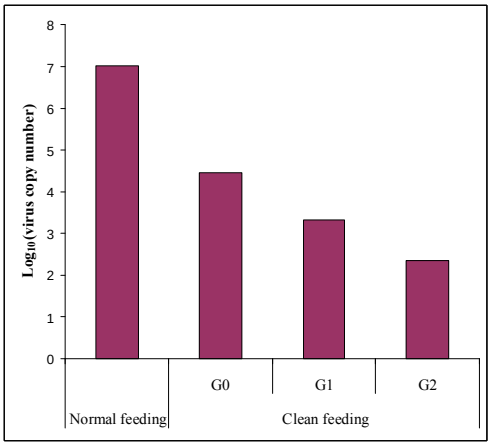

Figure 10. Virus copy number (log_{10}) in *Glossina pallidipes* (Tororo) colony flies fed using the normal feeding protocol and flies fed on clean blood for three generations.

million virus particles / fly were observed in the normal fed colony, only around 100 virus particles / fly were found in the clean fed colony after three generations. Although this result is very promising for a virus management strategy, it cannot be used routinely in a large mass rearing facility in view of equipment and labour requirements. Clean feeding however, could be used as an intervention strategy to reduce the initial virus load in a highly infected colony prior to the implementation of other management strategies to block virus transmission by inhibiting virus infection (e.g., neutralizing the virus infection by virus specific antibodies) or blocking virus replication (e.g., antiviral drugs and/or RNAi) when the colony is returned to the normal feeding system.

3.1.3.6. Antiviral drugs

As reported previously the impact of two antiviral drugs (Acyclovir and Valacyclovir) on virus replication was analyzed by qPCR. From the results reported last year one concentration (300 µg/ml) of the antiviral drugs Acyclovir and Valacyclovir (which results in an acceptable productivity, mortality and reduction of GpSGHV prevalence level when used over one generation) was used to asses the impact of the antiviral drug treatment on *G. pallidipes* flies when used over three generations. The results indicate that, while in general Valacyclovir long term treatment did not affect the level of pupae production, with Acyclovir treatment a significant reduction in fly productivity was observed. An interesting observation was that the virus load was significantly reduced in all treatments, including the control, over three generation (**Figure 11)**. Most likely the virus was lost from all treatments due to the use of clean blood for this experiment, avoiding virus contamination from other infected flies.

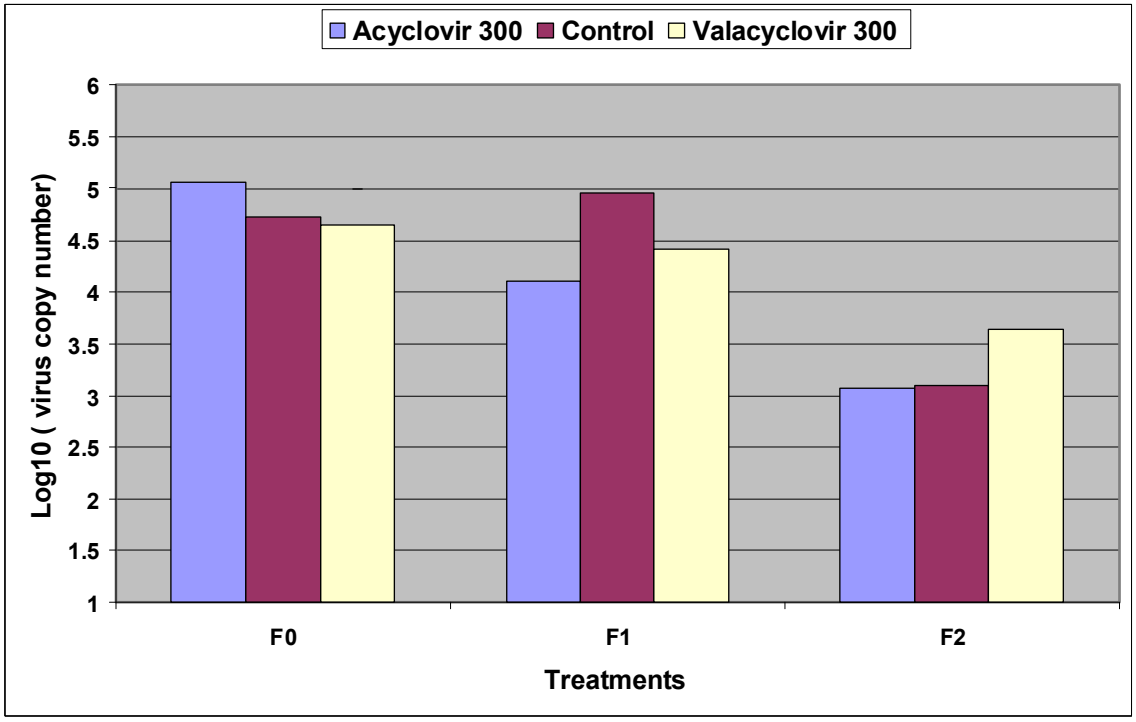

Figure 11. Impact of antiviral treatments on virus copy numbers over three generations.

To avoid this unwanted impact of clean feeding on the experimental objectives, the design of the experiment was changed. The clean feeding protocol was abandoned and the experimental blood, containing the antiviral drugs, was contaminated by feeding three cages of normally fed colony flies before feeding the experimental flies. In addition, rather than following individual generations separately, which is very difficult due to the overlap of the generations in tsetse, the data of the antiviral drug experiments were collected using normal weekly units. The results indicate that the productivity level was still better with Valacyclovir than Acyclovir (**Figure 12)**. A slight increase in mortality was observed with Valacyclovir.

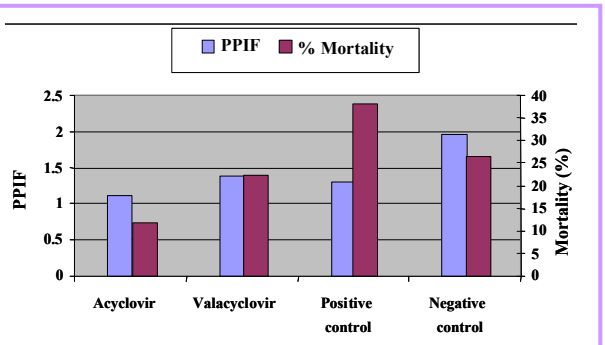

Figure 12. Impact of antiviral treatments on productivity and mortality of *Glossina pallidipes*. PPIF: pupae per initial female.

Flies treated with the antiviral drugs Acyclovir or Valacyclovir did not show any SGH syndrome (**Figure 13)**. Also, the qPCR analysis indicated a significant delay in the increase in virus load over time (**Figure 14)**.

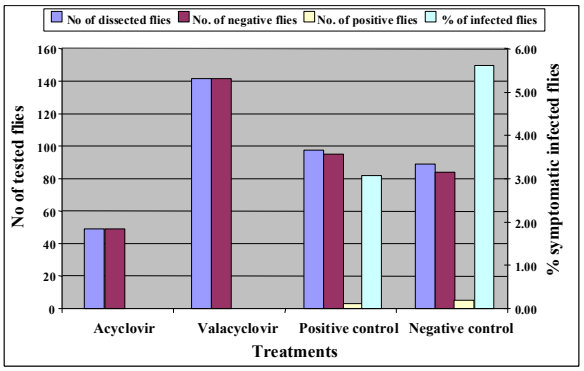

Figure 13. Impact of antiviral drug treatments on the prevalence of SGH.

Another approach was to develop an easy and quick method to test the direct impact of the antiviral drug treatment on virus replication. The virus particles collected from hypertrophied salivary glands under aseptic condition were injected into PCR negative teneral flies and the virus load was estimated by qPCR after different times. The results (**Figure 15)** show that the virus load reached around a million virus particles / fly at 28 days post injection. This test was used as a standard for virus replication. Feeding virus injected flies on blood treated with antiviral drugs resulted in a significant reduction in virus replication. Confirmation of these results and screening of more antiviral drugs are in progress.

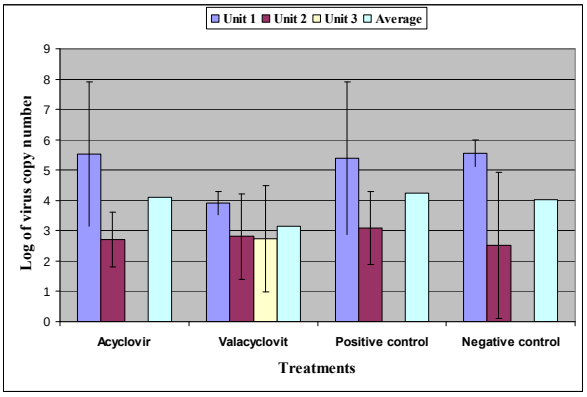

Figure 14. Impact of antiviral drug treatments on virus copy number. Positive control: feeding of previously contaminated blood. Negative control: feeding on clean blood.

3.1.3.7. RNAi experiments

RNAi technology can be used to reduce the expression of specific genes. An attempt was therefore undertaken to develop RNAi technology as a virus control strategy in addition to the antiviral drugs and antibody immunization approaches. As reported in the 2008 Activity Report, one gene was selected arbitrarily to test the application of RNAi in tsetse flies. The N-terminal, the C-terminal and the total sequence of the selected gene were cloned in a plasmid vector that was used to transform *E. coli*. Adding the non transformed bacteria to the tsetse blood diet had no impact on productivity and survival of flies.

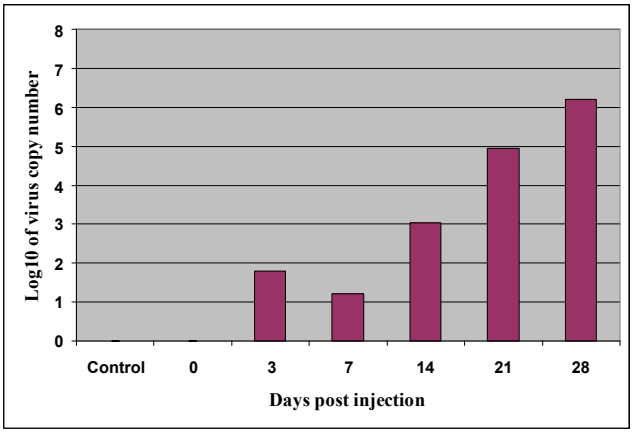

Figure 15. Increase of virus copy number in virus injected flies over time. (qPCR background value (0.35) in control and zero time was subtracted from all values).

The impact of RNAi treatments was tested over three generations. Whilst no negative impact on fly productivity and mortality was reported, a significant decrease in the virus load in the treated *G. pallidipes* was observed (**Figure 16**). But, as mentioned in the antiviral drugs experiments, a decrease in the virus load was also observed in the control flies. As a consequence, the experimental design was changed to contaminated feeding and weekly unit recording. Some technical problems were observed, like losing the recombinant plasmid after several generations of the bacteria, which required us to renew the bacterial transformation periodically. These problems have delayed this work somewhat.

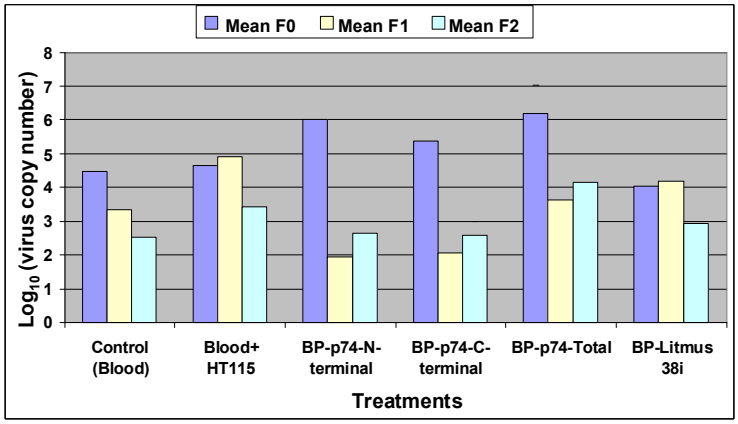

Figure 16. Impact of RNAi treatment on the virus copy number in F_0, F_1, and F_2 generations.

As seen in the earlier RNAi experiment no negative impact on productivity or mortality has been observed but also no significant reduction in the virus copy number has been found. Further work is in progress to confirm these results and to screen more virus genes for potential RNAi to inhibit the virus infection.

3.1.3.8. Neutralizing the virus infection with virus specific antibodies

Neutralizing virus infections with virus specific antibodies is well known as a strategy to manage virus infections. Although it was shown that the second step of the horizontal transmission (picking up the virus from contaminated blood by non infected flies) can be reduced by clean feeding, this method would be impractical in a large scale facility. Therefore, we tried to block the virus infection by neutralizing the virus released into the blood by feeding infected flies with virus specific antibodies. Six antibodies are being tested; two antibodies against the p74 protein produced by a commercial company, one antibody against each of the proteins expressed by the open reading frame (ORF) 10, 17 and 96 produced in collaboration with Prof. Just Vlak from Wageningen Agricultural University, The Netherlands and Prof. Max Bergoin from Montpellier University, France and one antibody extracted from rabbits used for tsetse feeding in CIRAD, Montpellier, France prepared by Prof. Max Bergoin.

All the antibodies were tested against GpSGHV virus particles by Western blot analysis and the results show that five of the antibodies react specifically to the virus particles **(Figure 17).** The antibodies extracted from the CIRAD rabbit was reactive against non-hypertrophied glands as well as purified virus particles. Experiments to assess the efficacy of the antibodies to neutralize and block virus infection are dependant on developing an effective artificial oral infection method. Initial experiments to use symptomatic flies as a virus source are in progress.

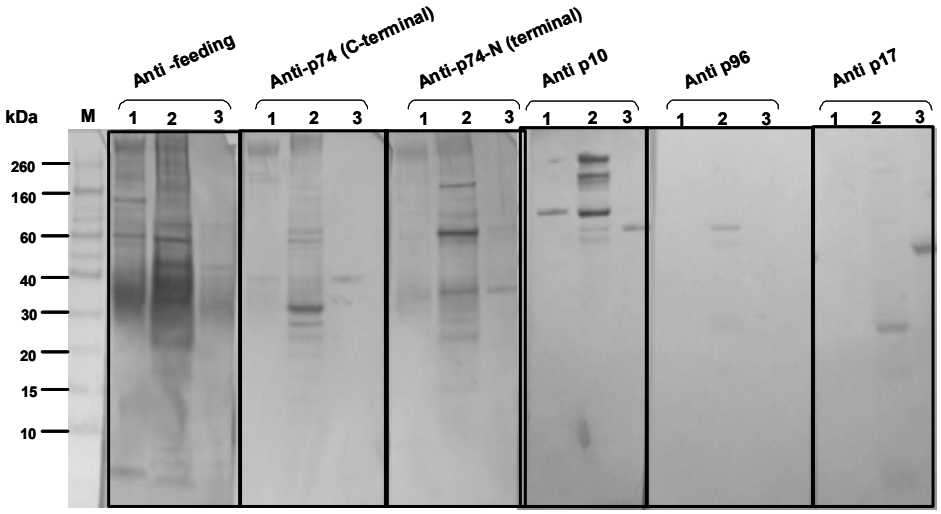

Figure 17. SDS-PAGE of non-infected *Glossina pallidipes* salivary gland cells (lane 1), hypertrophied *G. pallidipes* salivary glands (lane 2) and Nycodenz-purified GpSGHV virions (lane 3) subjected to Western blot analysis with rabbit antibodies against CIRAD anti-feeding antibodies, the C- and N-terminal peptides of SGHVp74 proteins, SGHV010 (C-terminal), SGHV096, SGHV017. The antibodies were diluted 1: 500. M denotes Norvex® Sharp Standard.

3.1.3.9. Analysis of the ultra-structure of GpSGHV particles with cryo-electron microscopy

In order to collect the maximum information about the ultra-structure of the virus particles, a collaboration has been started with Prof. Max Bergoin from Montpellier University and Prof. Robert Drillian, Danièle Spehner and Igor Orlov from the Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch-Graffenstaden, Strasbourg, France, to study the structure of the virus particles with cryo-electron microscopy. As presented in **Figure 5**, the virus particles look flat and flexible. Work is in progress to collect more information about the structure of this new virus.

3.1.3.10. Completion of the genome sequence of the GpSGHV Ethiopian isolate

Although a large part of the genome of GpSGHV isolated from the Ethiopian *G. pallidipes* colony established in Kaliti, Ethiopia, was sequenced, some regions remained unsequenced with the traditional Sanger sequencing method. To sequence these regions and to confirm the sequence of the region previously sequenced we re-sequenced all of the genome using the 454 sequence method that was used to sequence the Uganda strain. The 454 sequencing was successful and the sequence of the Ethiopian strain is now completed. The sequence data are currently undergoing quality control and analysis.

3.1.3.11. Attempt to clone the complete genome of GpSGHV into Bacmid

The difficulty of harvesting GpSGHV particles from hypertrophied salivary glands for experimental work and the absence of a permissive cell culture line for virus production means that much basic information about the virus is still not available and will continue to be difficult to collect. The construction of a recombinant Bacmid plasmid containing the complete genome sequence of SGHV would permit production of the viral sequence in a bacterial culture in sufficient quantity, which will create opportunities for more fundamental research (e.g., production of virus particles tagged with GFP or analysis of the gene function by site directed mutation). Cloning the complete genome of SGHV into the BAC required the following:

1- Modifying the commercial BAC (pBleoBAC II) by insertion of the restriction enzyme site of the required restriction enzymes (restriction enzyme which cut only one time in GpSGHV *Pme* I and *Asi*S I). This step was carried out at the Insect Pest Control Laboratory in collaboration with Prof Max Bergoin to produce the plasmid pBleoBAC11-M.

2- Extracting the intact DNA from the virus and digesting it with the selected restriction enzyme and clone it with the modified BAC.

To avoid shearing the DNA during the extraction process, it is recommended to make the DNA extraction and restriction enzyme digestion in an agarose block followed by migration of the digested DNA by pulse field gel electrophoresis (PFGE). As this electrophoresis system and the required expertise were not available in Seibersdorf, it was run in Montpellier. Preparation of the virus samples was started in Seibersdorf and hand carried to Montpellier. The samples were washed and digested in the block; then the DNA was separated by PFGE **(Figure 18)** in collaboration with Dr. Corinne Teyssier and Prof. Estelle Jumas-Bilak, Laboratoire de Bactériologie, Faculté de Pharmacie, Université de Montpellier, where the necessary equipment was available. The work to clone the DNA extracted from the agarose gel in the modified pBloeBACII-M plasmid is in progress.

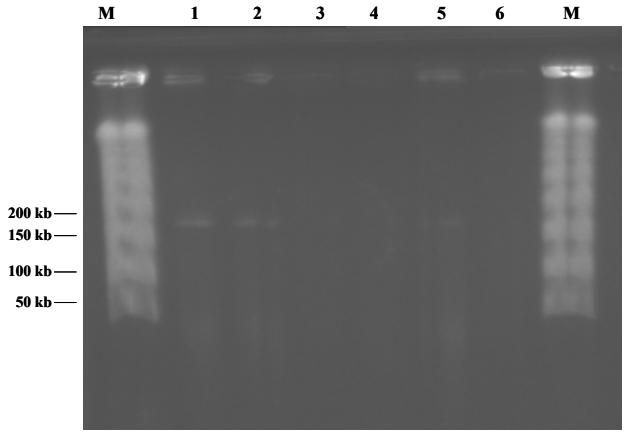

Figure 18. Low melting agarose block treated with SDS lysis buffer. 1 and 2: non purified virus digested with Pme I and AsiSI,, 3 and 4: purified virus digested with Pme I and AsiSI, resp., 5 and 6 non digested DNA of non purified and purified virus, resp. M : DNA ladder of concatemer of lambda virus.

3.1.3.12. Revised virus classification

As reported last year, a classification proposal for GpSGHV was submitted to the International Committee on Taxonomy of Viruses (ICTV) suggesting a new virus family, Hytrosaviridae, for these new viruses **(Figure 19).** In the taxonomic structure of this family we did not propose any genera and left the two species GpSGHV and *Musca domestica* salivary gland hypertrophy virus (MdSGHV) unassigned in the family. The ICTV rules require at least one genus to be erected and due to the significant differences between the two viruses in genome and virus particle length, and the specific biology, we have now proposed two genera (Glossinavirus and Muscavirus) in the Hytrosaviridae family. A potential species *Merodon equestris* salivary gland hypertrophy virus (MeSGHV) was proposed but remains unassigned to a genus.

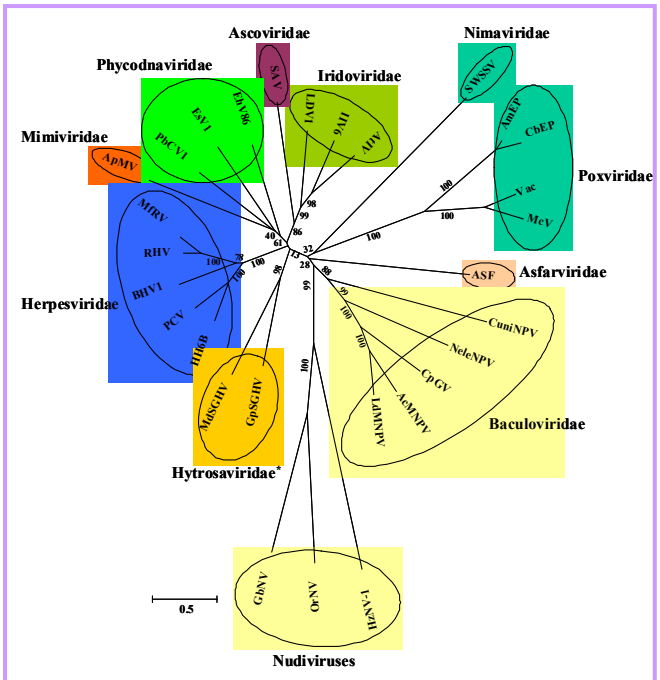

Figure 19. Phylogenetic analysis tree showing the genetic distance of Hytrosaviridae family from other large double stranded DNA viruses.

3.1.4. Field Cage Tests of the Effect of Various Factors on the Mating Performance of Male Glossina palpalis gambiensis

3.1.4.1. Effect of age on mating performance

The effect of age on male *Glossina palpalis gambiensis* competiveness was investigated. The mating competitiveness of males 3-, 6- and 9-days after adult emergence was assessed. Flies received a blood meal the day before the field cage observation, and the marking of these age groups was also done a day before field cage observation. The unmarked group was also subjected to the low temperature experienced during marking. A walk-in field-cage was used in order to approximate as closely as possible the actual field scenario during sterile insect release programmes. The field cage was erected inside a green house under natural light without environmental controls.

Observations were conducted from 0900h and ended at 1200h. Temperature ranged from 18°C at the start to 36°C at the end, humidity ranged from 67% down to 26%. There was hardly any flight activity below 20°C with the exception of flight immediately after release. Most flies rested in darker spots in the lower portions of the cage when temperature breached 27°C. It was shown that 9- and 6-day old males were significantly more competitive than 3-day old males, with the greatest number of mating pairs involving the oldest males and the youngest having the least number of pairs.

The ability of *G. p. gambiensis* to inseminate was not age dependent, and insemination occurred in most females that mated regardless of male age, however, 3-day old males transferred lower volumes of seminal contents. Mating duration was significantly shorter for 3-day old males, whereas it was the same for 6- and 9-day old males. We had not anticipated such a delay in the development of mating performance, which is more like that of *G. pallidipes*, and the proposed timing of field releases of sterilized males should be reviewed.

3.1.4.2. Effect of chill holding of male pupae on mating performance

Field cage tests were carried out with *G. p. gambiensis* to assess the impact of delaying male adult emergence by chilling to simulate the procedure needed to transport male pupae from Burkina Faso to Senegal. For the control, normal colony emergence of males was used. For the chilled treatment, male pupae collected after female emergence was completed, were chilled at 12.5°C for seven days to inhibit emergence and then returned to normal colony conditions for emergence. The tests were carried out with males 3, 6 and 9 days post emergence. Competitiveness tests were carried out between control males and males from the chilled treatment of the same post emergence age, i.e. 3 days control vs. 3 days chilled, 6 days control vs. 6 days chilled, and 9 days control vs. 9 days chilled. The males competed for mating opportunities with 3-day old females that emerged under normal colony conditions. Observations were carried out in a field cage erected in the insectary building under fluorescent lights, temperature 24°C, relative humidity 60%. The observation time was 09h00 to 12h00 on each day. There were significantly more mating pairs recorded for older males than the younger males (a similar trend as observed in the direct competition tests above) irrespective of whether they were chilled or not (**Figure 20**). Premating time was slightly shorter on average for 3 days chilled males (63 minutes) compared to control males (88 minutes) whereas it was longer for 6 and 9 days chilled males (56 minutes and 32 minutes respectively) compared to the control males (51 and 25 minutes respectively). Overall the 9 days old mated earlier than the 3 and 6 day-old males. The mating duration was on average

similar for 6 and 9 day-old males for both treatments (87-89 minutes for control and 75 to 83 minutes for chilled) and slightly shorter for 3 day-old males, 69 minutes for the control and 59 minutes for the chilled males.

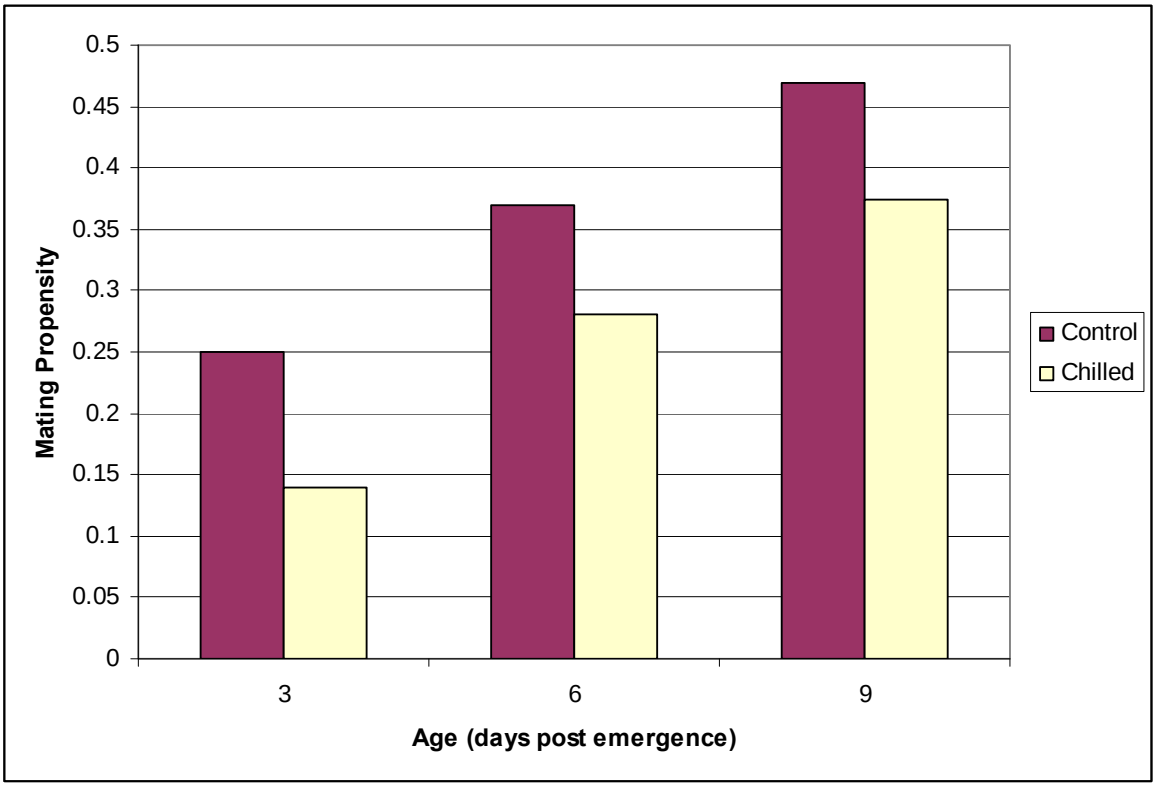

Figure 20. The proportions of male *Glossina palpalis gambiensis* that mated with 3 day-old females.

Half of the mating pairs involving 9 day old males were initiated within the first ten minutes of observations, whereas it was up to thirty minutes and up to one-and-half hours for 6 day- and 3 day-old males respectively. Mating duration was nearly the same for control and chilled flies.

It seems 12.5°C is very close to the limit at which the pupae can be kept for seven days without emerging. Flight activity is more frequent for the older males than the younger chilled males

3.1.4.3. Effect of irradiation on mating performance

In a similar field cage environment males irradiated as adults with 70, 110 and 130 Gy were out-competed by unirradiated males from the same Burkina Faso stock. Equal numbers of 7 day-old irradiated and unirradiated males were presented with mating opportunities with 3 day-old females. Unirradiated males constituted one third of the total number of pairs formed, males irradiated with 70 Gy were involved in one-sixth of the total and the remainder were equal proportions of males irradiated with 110 Gy and 130 Gy. Spermathecal fill for females that mated with irradiated males was slightly lower than for females that mated with

unirradiated males. The time that elapsed from release to initiation of mating was longer for irradiated males and the duration of copulation marginally shorter in irradiated males.

3.1.4.4. Controlled environment room for field cage

The field cage tests in the constant environment room confirmed the suitability of the selected temperature and humidity levels for mating competitiveness tests with *G. p. gambiensis*. Although the fluorescent light was of lower intensity than natural light, the tsetse fly activity in the cage was similar to that observed in the greenhouse. The flies responded to movement by the observer and the preferred landing sites were the darker areas of the cage, for instance the PVC frame, the junction of the roof with the side wall and the ground with the side wall. However, fewer flies landed on the tree in the constant environment room (insectary) than when the cage was in the greenhouse. The proportions that mated were also similar in both environments.

3.1.4.5. Morphometric analysis of Glossina palpalis gambiensis *wings*

Wings of *G. p. gambiensis* collected from four populations in Senegal in May 2009 were subjected to morphometric analysis. Images of the wings were captured using a Dino-Lite® microscope and landmark recording was done using the software COO by Jean-Pierre Dujardin. The coordinate data were used for procrustes fit. A procrustes analysis of variance and regression analysis on centroid size were carried out using the software MorphoJ version 1.01a.

The results show the biggest differences between Parc de Hann and Diacksao Peul and between Parc de Hann and Sebikotane fly groups. There are lesser differences between Pout - Diacksao Peul, Pout - Parc de Hann, Pout - Sebikotane groups. The most similar fly samples are Sebikotane and Diacksao Peul. There is no significant difference between these two groups after the permutation test.

An analysis of wings of *G. p. gambiensis* from the Seibersdorf colony (originally obtained from Burkina Faso) from February 2009 to June 2009 was also carried out using the same software. In February the size of the Seibersdorf colony flies was more variable but by May – June the variability was similar to the sample from Senegal.

3.1.5. Irradiation and Dosimetry

3.1.5.1. Final development and evaluation of the X-ray irradiator

The characterization of the RadSource RS2400™ X-ray irradiator has been reported in the last two Activities Reports. During 2009 we were able to complete this work, and as a result of the increasing difficulties in shipping Co^{60} gamma sources, the Agency's Technical Cooperation Department has ordered a number of units for use in Member States.

At the end of 2008 we had determined the depth dose relationship and tested several spectral hardening filters. The final step was to perform a complete dose mapping (**Figure 21**) to determine the dose uniformity and to identify any remaining areas of high or low dose.

The dose map shows that we have substantially achieved our objective of obtaining a dose uniformity ratio (DUR) of 1.3:1. The map shows that the tube is not exactly concentric to the

canisters and that the length of the canisters needs to be reduced by some 5 mm at the top of **Figure 21**.

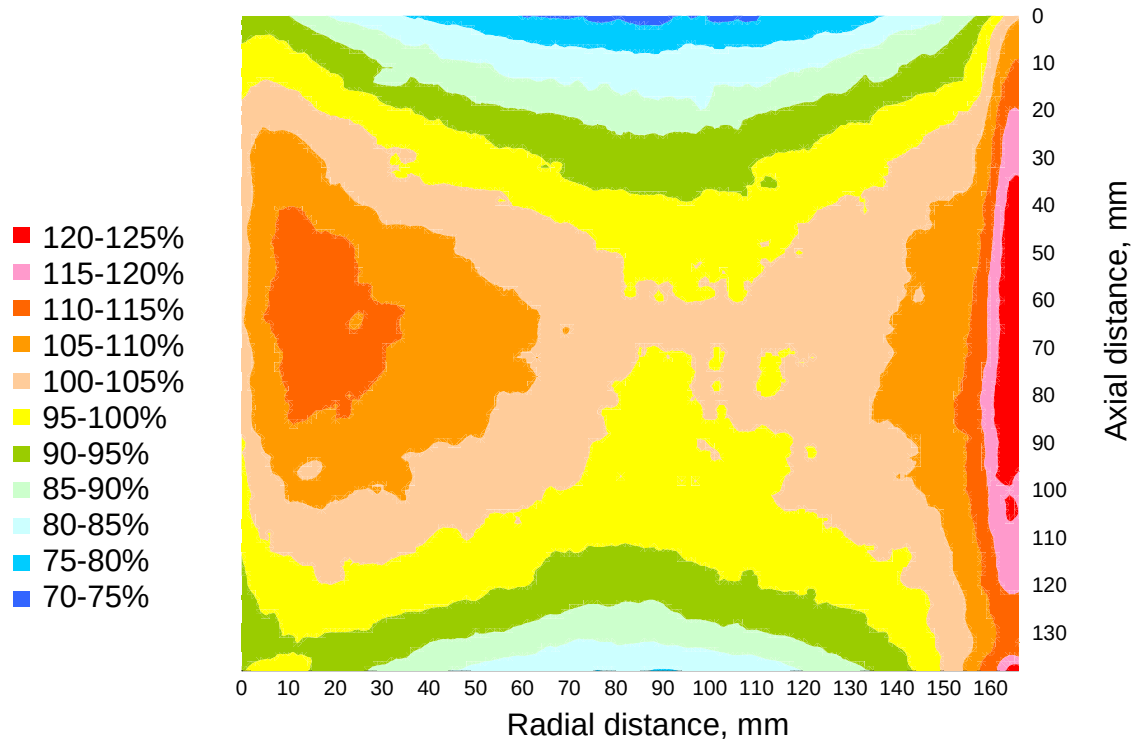

Figure 21. Dose map of one canister filled with instant rice. The other four canisters were also filled with instant rice. Mapping was done using Gafchromic HD810 film scanned on an Epson 10000 XL scanner at 50 dpi and 48 bits per pixel. The green channel information was extracted and averaged over a 2 x 2 pixel square to give a final resolution of 1 mm. Computed dose values were normalized to the median pixel value.

3.1.5.2. Dose comparison scheme

Following a trial in 2008, the Insect Pest Control Laboratory initiated a dose comparison scheme in 2009 for SIT programmes. The purpose of the scheme is to provide a means for SIT programmes to check that their insects are receiving the appropriate dose to ensure an adequate level of sterility without the negative consequences of over or under-dosing. The scheme is, however, not for calibration, which must be done with suitable transfer standard dosimeters supplied and read by an accredited laboratory.

The scheme operates by supplying a set of Gafchromic dosimeters, 1 x 1 cm, consisting of three test dosimeters and six controls, three unirradiated and three irradiated to 100 Gy, sealed in two small polythene pouches to protect them. The recipient should include the test dosimeters in a routine irradiation at their reference location. When the dosimeters are returned to Seibersdorf they are read and the dose and confidence interval calculated. This is then reported back to the SIT programme so that they can compare this estimated dose with their intended target dose at the reference position.

When a programme has direct control of their own irradiation and dosimetry the scheme will confirm their results and give them more confidence in their own procedures and dosimetry. More importantly, perhaps, when a programme relies on another institute or organization to perform the irradiation and dosimetry for them it will provide an independent check on the dose to alert them to possible problems. The servicing institute or organization can then be approached to investigate more fully the situation and take corrective action if necessary.

The dose comparison scheme is being run free of charge at the moment and it is intended to send dosimeter sets approximately annually. Additional sets or more frequent supply can be arranged on request, particularly if an irradiation problem is identified. Twenty-three sets were sent in the first round in late 2009.

3.2. Fruit Fly Rearing and Quality Control

3.2.1. Studies on Species within the Bactrocera dorsalis *Complex*

There is increasing demand, world wide, to address the issue of fruit fly species complexes, i.e. fruit fly species with distinct similarities that have been grouped together. There is concern that some of these insect lines are not separate species but geographical variants. Their uncertain taxonomic status is having significant implications for international trade resulting in the establishment of trade barriers to important commercial fruit and vegetable commodities.

On the other hand, some insect populations grouped within the same species display different mating behaviours and/or times. This has important practical and economic implications for the effective use of the sterile insect technique (SIT) against such fruit fly pests.

Regional insect rearing facilities that would produce sterile insects for SIT programmes in various regions or countries are desirable as this would make the SIT component of these programmes more cost-effective. It is of prime importance, therefore, to the success of these regional area-wide integrated pest management programmes that mass-reared flies are compatible with the target native fruit fly pest populations.

A call for research proposals for a new Coordinated Research Project (CRP) on this issue was made in late 2009 with considerable interest from many Member States to participate. The main targets of research will be the *Bactrocera dorsalis* (Asia and Pacific Ocean, Africa), *Anastrepha fraterculus* (Mexico, Central and South America), and *Ceratitis rosa* (Africa and Indian Ocean) species complexes. Cryptic (i.e. insects whose specific status is questionable) pest fly species within each complex will be laboratory reared and mating propensity, compatibility and competitiveness assessed in laboratory and field cages. In addition, cytogenetic, molecular and pheromone differences between species will be assessed. The desired output of the CRP will be a clear description of biological characteristics of the different cryptic populations or species within each complex that will greatly facilitate and enhance the implementation of area-wide integrated pest management (AW-IPM) programmes that include an SIT component.

Laboratory colonies of several pest fruit fly species from the *B. dorsalis* species complex have been established at the Insect Pest Control Laboratory. Preliminary testing of interspecific hybridization has been carried out. The following species are currently being tested at Seibersdorf:

- *Bactrocera dorsalis* (laboratory-reared, originally from Thai mangoes)
- *Bactrocera invadens* (laboratory-reared, originally from Kenyan mangoes)
- *Bactrocera philippinensis* (laboratory-reared, originally from Philippines mangoes)
- *Bactrocera carambolae* (laboratory-reared, originally from Suriname carambolas)

3.2.1.1. Laboratory hybridization tests

For these experiments, laboratory cultures of the first three species were reared on the same larval rearing medium, the standard Seibersdorf diet which is based on wheat bran. Adult flies were kept under the same laboratory room conditions as each other (photoperiod 14h:10h

light:dark, RH 60% and temperature 25 ± 1ºC). Flies were sexed one day after adult emergence. Flies were kept in small Plexiglass cages (17 cm x 17 cm x 12 cm) with food (4 parts white crystalline sugar mixed with one part protein hydrolysate) and water. All flies were the same age (12 days) at the commencement of the experiments. Five flies of each sex were put in each cage. Three replicate cages per crossing were set up. Crosses between *B. dorsalis* and *B. invadens* have been reported previously (see Activities Report 2008). The *B. carambolae* colony is not yet up to the scale where hybridization trials can be conducted. The following crosses were made:

- *B. philippinensis* ♂×*B. philippinensis* ♀
- *B. dorsalis* ♂×*B. philippinensis* ♀
- *B. philippinensis* ♂×*B. dorsalis* ♀
- *B. invadens* ♂×*B. philippinensis* ♀
- *B. philippinensis* ♂×*B. invadens* ♀

Eggs were collected using a small, pin-pricked plastic container (drink bottle) with a few drops of commercial guava juice inside. The juice was used to stimulate oviposition. This egg collection device was kept in the cage for four hours after which the egging devices were removed from their cages and eggs were collected by washing out into beakers using a fine stream of water from a wash bottle. All collected eggs were counted and incubated for 24 hours at 26ºC and RH 60%. After 24 hours, eggs were transferred onto carrot powder based artificial larval diet. The egged diet was kept in an incubation room at 25ºC and RH 85% for 72 hours. After 72 hours the larval diet was moved to the maturation room and mature larvae were collected in damp vermiculite. Eggs were collected on six consecutive days from the first day of oviposition. Data on the numbers of egg oviposited each day, their fertility and the percentage of fertile eggs that produced pupae (egg to pupal recovery) were recorded.

In the laboratory hybridization tests all attempted hybridizations were successful. That is, under laboratory conditions, each pair of interspecific parents mated with each other and produced viable offspring. **Figure 22** shows that there were no differences in egg fertility between the crosses assessed, except that for *B. invadens* males crossed with *B. philippinensis* females the egg fertility was significantly lower than for the others ($p < 0.05\%$).

3.2.1.2. Field cage tests

Field cage trials with trees are continuing on all hybridizations mentioned above. During 2009 field cage tests were carried out with *B. dorsalis* and *B. invadens* under simulated natural conditions. These tests were done in a set of four field cages located at the Insect Pest Control Laboratory inside a temperature controlled (25 ± 3°C) greenhouse under natural light conditions. Three tests were done.

1. The first tested competition between *B. invadens* males and *B. dorsalis* males for *B. dorsalis* females (in one cage) or *B. invadens* females (in a second cage). Here 20 male *B. invadens* + 20 male *B. dorsalis* were exposed to 20 female *B. invadens* in one cage and 20 male *B. invadens* + 20 male *B. dorsalis* were exposed to 20 female *B. dorsalis* in a second cage. Two replicates of this experiment have been carried out to date.

2. The second experiment tested the likelihood of hybridization when both sexes of both species were exposed to each other in one field cage. Here 15 male *B. invadens*, 15 female *B.*

invadens, 15 male *B. dorsalis* and 15 female *B. dorsalis* were all placed into a field cage together. They had been painted the day before the experiment as described above. Mating couples were collected and identified as to species. Four replicate cages were used. Later this test was repeated but with 20 individuals of each sex / species instead of 15. Again four replicate cages were used. The order in which mating pairs formed was also recorded.

3. The third test assessed whether or not male F_1 hybrids would mate, under simulated field conditions, with their male and female parental lines. Here one cage had 20 male *B. dorsalis*, 20 female *B. dorsalis*, 20 male F_1 (male *B. invadens* × female *B. dorsalis*) and 20 male F_1 (male *B. dorsalis* × female *B. invadens*). A second cage had 20 male *B. invadens*, 20 female *B. invadens*, 20 male F_1 (male *B. invadens* × female *B. dorsalis*) and 20 male F_1 (male *B. dorsalis* × female *B. invadens*). Two replicates of this experiment have been carried out to date.

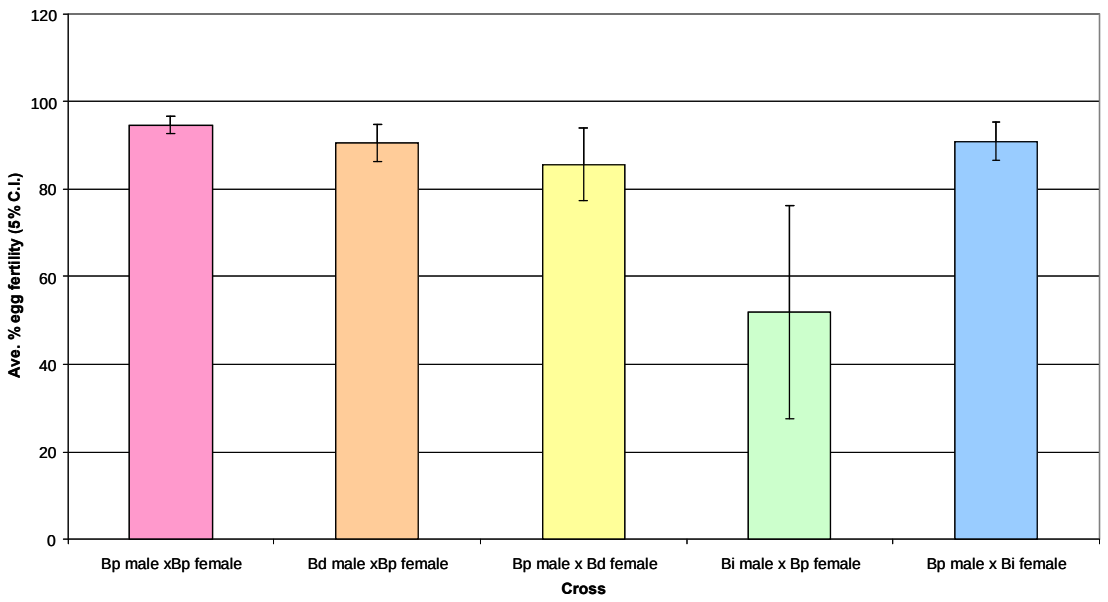

Figure 22: Fertility of eggs produced by interspecific crosses. Bd: *Bactrocera dorsalis*, Bi: *Bactrocera invadens*, Bp: *Bactrocera philippinensis*.

In all tests, prior to releasing into cages, virgin adult males and virgin adult females of each species were "painted" by restraining individual adults in a flattened mesh bag and then dotted with a small mark of water-based paint. Different species were painted with different colours to facilitate species identification. Virgin males were placed into their field cages 30 minutes prior to the release of virgin females. Mating couples were collected in small capped bottles and their species determined the next day.

In each case there was some interspecific mating between *B. dorsalis* and *B. invadens*. In the first field cage tests (**Table 3)** *B. invadens* females seemed to prefer to mate with *B. dorsalis* males in both replicates over *B. invadens* males (Test A). *B. dorsalis* females preferred to mate with *B. dorsalis* males in Test B.

Table 3. Field cage tests assessing competition between males of one species with males and females of the other species.

TEST A: 20 Bd males with 20 Bi males and 20 Bi females		
Date of rep.	**Cross type**	**Number of pairs**
25-Aug-2009	Bd male × Bi female	12
	Bi male × Bi female	7
07-Sep-2009	Bd male × Bi female	12
	Bi male × Bi female	5
TEST B: 20 Bi males with 20 Bd males and 20 Bd females		
Date of rep.	**Cross type**	**Number of pairs**
25-Aug-2009	Bi male × Bd female	7
	Bd male × Bd female	13
07-Sep-2009	Bi male × Bd female	5
	Bd male × Bd female	15

Bd = *Bactrocera dorsalis*; Bi = *Bactrocera invadens*

Hybridization also occurred in the second test, where equal numbers of both sexes of one species were exposed to both sexes of the other species. There was some variation between replicate 1 and replicate 2: while the Relative Isolation Index (RII) was high in replicate 1, indicating that each species preferred to mate intraspecifically (**Table 4**), the RII was lower in replicate 2. Here the number of interspecific matings was similar to the number of matings between male and female *B. invadens* (but fewer than those between male and female *B. dorsalis*). In total, similar numbers of male and female *B. dorsalis* and male and female *B. invadens* (especially in Rep. 1) participated in matings. When the first 15 matings were assessed (**Table 5**) the response was different. During

Table 4. Field cage tests of both sexes of both species with mating compatibility indices based on all observed matings in each of 4 cages/ replicate.

	Rep 1	**Rep 2**
Bd male × Bd female	31	44
Bi male × Bi female	33	23
Bi male × Bd female	18	22
Bd male × Bi female	23	25
Total	**105**	**114**
Total no. of Bi males mated	51	45
Total no. of Bi females mated	56	48
Total no. of Bd males mated	54	69
Total no. of Bd females mated	49	66
Relative Isolation Index (RII)	**2.47**	**1.84**
Isolation Index (I)	**0.64**	**0.70**
Isolation Index (ISI)	**0.22**	**0.18**

Bd = *Bactrocera dorsalis*; Bi = *Bactrocera invadens*

this period most matings were between the males and females of *B. dorsalis* (intraspecific) and between male *B. dorsalis* and female *B. invadens* (interspecific). The RII, here, was closer to unity (random mating between species). *B. dorsalis* males tended to be the dominant mating partner whether with a female *B. dorsalis* or female *B. invadens* during this time. Fewer *B. invadens* males mated during this time (**Table 5**) but, by the end of the evening similar the number of male *B. invadens* who had mated was similar to that of the others (**Table 4**).

Table 5. Field cage tests of both sexes of both species with mating compatibility indices based on the first 15 matings in each of 4 cages/ replicate.

	Rep 1	**Rep 2**
Bd male × Bd female	23	31
Bi male × Bi female	7	4
Bi male × Bd female	8	9
Bd male × Bi female	22	16
Total	**60**	**60**
No. of Bi males mated	15	13
No. of Bi females mated	29	20
No. of Bd males mated	45	47
No. of Bd females mated	31	40
Relative Isolation Index (RII)	**0.91**	**0.86**
Isolation Index (I)	**1.00**	**0.71**
Isolation Index (ISI)	**0.00**	**0.17**

Bd = *Bactrocera dorsalis*; Bi = *Bactrocera invadens*

In the third tests, when male F_1 hybrids (from both male *B. invadens* × female *B. dorsalis* and male *B. invadens* × female *B. dorsalis*) were exposed to male and female *B. dorsalis* (**Table 6**: Test A) there was a preference for intraspecific mating. However when these males were exposed to male and female *B. invadens* there was no preference for intraspecific mating (**Table 6**: Test B).

Table 6. Field cage tests assessing competition between hybrid (*B. invadens* X *B. dorsalis*) males with males and females of parental lines.

TEST A: 20 *di* males with 20 *id* males, 20 Bd males and 20 Bd females

Date of rep.	**Cross type**	**Number of pairs**
25-08-2009	*di* male × Bd female	3
	id male × Bd female	4
	Bd male × Bd female	13
26-08-2009	*di* male × Bd female	4
	id male × Bd female	3
	Bd male × Bd female	13

TEST B: 20 *di* males with 20 *id* males, 20 Bi males and 20 Bi females

Date of rep.	**Cross type**	**Number of pairs**
25-08-2009	*di* male × Bi female	3
	id male × Bi female	8
	Bi male × Bi female	8
26-08-2009	*di* male × Bi female	9
	id male × Bi female	8
	Bi male × Bi female	2

id = F1 Hybrid of Bi male X Bd female; *di* = F1 Hybrid of Bd male X Bi female;
Bd = *Bactrocera dorsalis*; Bi = *Bactrocera invadens*

Our data have indicated that in the laboratory under no-choice conditions interspecific crossing between *B. dorsalis*, *B. invadens* and *B. philippinensis* can occur and the resultant F_1 hybrids are fertile. When tested under simulated field conditions (in field cages with trees) there was a tendency to a preference for intraspecific mating overall. Nevertheless, there is a trend for male *B. dorsalis* to mate early without much discrimination between female *B. dorsalis* and *B. invadens*. The suggestion is that there may be some temporal difference between the time of day *B. dorsalis* mates and when *B. invadens* mates, although it may be by no more than 30 to 60 minutes or possibly less. It is possible in this type of experiment, that as the number of *B. dorsalis* females that are available to *B. dorsalis* males diminishes they then mate with *B. invadens* females. As the period of the day for *B. invadens* mating approaches there are few female *B. invadens* left for male *B. invadens* to mate with. This problem can be overcome by replacing flies whenever a couple is removed.

It is not known if there is, in reality, such a temporal difference in mating time between these two species. It is possible that the observed differences are due to artefacts induced by laboratory-rearing. More replication of these experiments is underway.

3.2.1.3. Comparison of egg size and pupal weight

Preparatory to conducting assessments of the dimensions of various morphological characters of flies within the *B. dorsalis* species complex some trials on comparing the dimensions of the eggs of *B. dorsalis*, *B. carambolae*, *B. invadens* and *B. philippinensis* were carried out. This was a preliminary trial designed to test the possibility of using egg dimensions to aid in distinguishing between species within the *B. dorsalis* complex. Eggs were collected from the four laboratory-reared fly species and measured, using the Motic software (camera and microscope connected to a PC), the length, maximum width and length of perimeter for 20 eggs from each species. Calibration was made against a standard 2 mm circle and was tested before and after each set of measurements was made to ensure consistency. Five samples of 100 pupae from each species were weighed on an analytical balance and the average weight of a single puparium was estimated for each species. All test insects were reared under the same climatic conditions (temperature 25°C, RH 65% and light regime 14h:10h light:dark) in the same larval rearing medium (standard Seibersdorf diet based on wheat bran). Approximately equal volumes of eggs were seeded onto the larval rearing medium. Eggs were collected using the same type of egging device as described above. Pupae from each species were collected in damp sawdust and kept at 26°C and then weighed on the eighth day post adult emergence. All were treated in the same way. As a comparison, 20 eggs from each of the following species were also measured: *Anastrepha fraterculus*, *Ceratitis capitata* and *Bactrocera oleae*.

There were some significant differences in egg dimensions measured in these experiments between the species tested (**Table 7**). Based on the data recorded in this experiment, at the 5% level of significance, eggs of *B. carambolae* are significantly longer and narrower than those of *B. philippinensis* with a significantly longer perimeter than both *B. invadens* and *B. dorsalis*. *B. philippensis* eggs are also significantly wider than *B. invadens* and there is only a small overlap (within the 5% level of significance) with *B. dorsalis* eggs. Nevertheless, *B. dorsalis* and *B. invadens* can not be separated on any egg dimension value tested here.

When grown under identical conditions there were significant (5% CI) differences between pupal weights of the four species. *B. invadens* puparia (13.76±0.18 mg) were significantly

heavier than all others tested. There was no significant difference between *B. philippinensis* (12.92±0.14 mg) and *B. carambolae* (12.69±0.13 mg) but all were significantly heavier than *B. dorsalis* (12.02±0.14 mg).

Table 7. Comparative dimensions of eggs in mm (± sd) of some species within the *Bactrocera dorsalis* species complex (n=20). Within column, means with different letters are significantly different at the 5% level of significance

Species	Length	Width	Perimeter
B. carambolae	1.210 (0.051) a	0.261 (0.014) b	2.744 (0.156) a
B. philippinensis	1.151 (0.073) b	0.281 (0.014) a	2.611 (0.214) ab
B. invadens	1.180 (0.050) ab	0.267 (0.009) b	2,634 (0.086) b
B. dorsalis	1.187 (0.059) ab	0.269 (0.014) ab	2.614 (0.113) b

When compared with other species, egg dimensions of the *B. dorsalis* complex species tested here were smaller than those of *Anastrepha fraterculus* eggs and larger than those of both *Ceratitis capitata* and *Bactrocera oleae* eggs (**Table 8**).

Table 8. Comparative dimensions of eggs in mm (± sd) of some pest fruit fly species (n=20).

Species	Length	Width
Anastrepha fraterculus	1.351 (0.045)	0.259 (0.149)
Ceratitis capitata	0.983 (0.047)	0.236 (0.120)
Bactrocera oleae	0.726 (0.036)	0.231 (0.114)

There were some significant differences observed in egg dimensions between the four species tested within the *B. dorsalis* complex. The degree of variation in egg size within species in nature is not known but it is suspected to be low (**Figure 23**). For example, *Bactrocera tryoni* eggs are reported to not vary in size with varying adult size and quality. If this is the case then measurements of egg dimensions may be useful in aiding identification of pest fruit fly species.

The weight of puparia does vary considerably depending on host quality, interlarval competition for host resources, temperature and RH during development, pupal age and other factors. Although same volumes of eggs of each species were seeded onto the larval rearing media, variation between species in egg fertility may impact on the number of larvae competing within the diet for nutrients. We consider this unlikely as the volume of eggs per unit volume of medium is generally very low so resource limitation within the range of likely fertility rates between species would not impact adversely on pupal weight. There appears to intrinsic differences between these species in pupal weight but this measure is unlikely to be useful for identification purposes.

Figure 23. Fruit fly eggs. Left to Right: *Anastrepha fraterculus*, *Bactrocera dorsalis*, *Ceratitis capitata*, *Bactrocera oleae.*

3.2.2. Studies on the Development of Mass-rearing for Bactrocera oleae

The olive fly *Bactrocera oleae* (Rossi) is the key pest in all regions cultivating olives in Europe, the Middle East and Northern Africa as well as being a significant economic pest in some parts of the USA which this pest has recently invaded. Research over the last three decades has been targeting the suppression of *B. oleae* (among other pest insect species) populations through the use of the sterile insect technique (SIT). The SIT has the advantages that it has low impact on the environment and human health and it is target-specific in that no beneficial organisms are affected adversely. The effectiveness of the SIT when applied as a component of area-wide integrated pest management programmes depends on the ability to produce large quantities of insects at a reasonable cost but also the ability to mass-produce sterile insects that can effectively compete with wild insects to induce sterility into the target population. Cost of production and release and the quality of released insects are essential elements to a successful SIT programme.

Technicians at the Insect Pest Control Laboratory have been studying ways of reducing the costs of *B. oleae* mass-production and increasing the effectiveness of rearing techniques. Parts of these studies have been reported in previous annual Activities Reports.

3.2.2.1. Optimum sex ratio for egg production

One of the shortcomings of current *B. oleae* laboratory production is that the optimal number of fertile eggs per female has not been reached consistently. Factors that affect the volume of eggs produced include climatic factors such as temperature, relative humidity and lighting conditions; and those related to female fecundity such as access to food, water and space, quantity and type of food, access to oviposition site, access to males, access to undisturbed mating site, quality of males and females, and many more. The experiment described here is one where various ratios of males to females were housed in cages and egg production monitored over time.

Small, clear plastic cages measuring 4.5cm x 4.5cm x 9.5cm long (with internal volume of approximately 175 cm^3 and an internal surface area of approximately 195 cm^2) were used in this experiment (see **Figure 24**). The cage comes apart in two pieces – one 6.5 cm long and the other 3 cm long (the two fit into each other to a depth of 0.5mm). The larger portion of the cage was used to house adult *B. oleae* flies. Therefore the volume and surface area available to the flies in these experiments were 116 cm^3 and 144 cm^2 respectively. Before fitting the two portions together the open end of the larger portion was covered with a square (5cm x 5cm) of waxed oviposition panel (fine terylene cloth coated with a mixture of beeswax, paraffin oil and paraffin wax). The cloth overlapped the sides of the cage so that when the

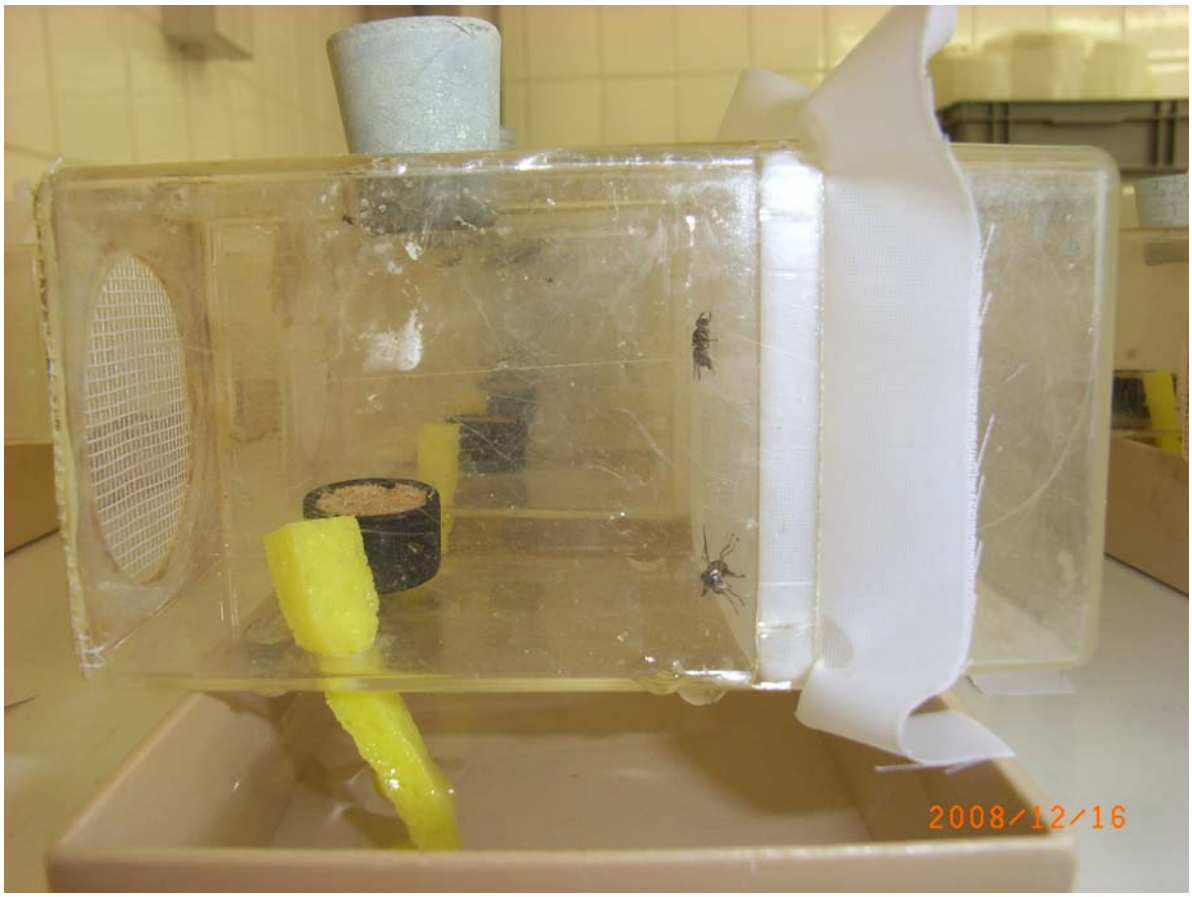

Figure 24. Small cages used for *Bactrocera oleae* experiments.

smaller portion was connected it held the cloth firmly inside the cage. The other end of the larger portion was also open to the air but covered with a fine mesh for cage ventilation purposes.

Various numbers of virgin *B. oleae* adults were placed into the cages (with waxed oviposition panels) and placed in an environment controlled room at 25°C and RH 65% with a light regime of 14h:10h light:dark. Ratios tested (male:female) were: 0:1, 0:2, 0:3, 0:4, 0:5, 1:1, 1:2, 1:3, 1:4, 1:5, 2:1, 3:1, 4:1, 5:1, 2:2, 3:3, 4:4, 5:5, 10:10, 15:10, 20:10, 1:10, 1:15 and 1:20.

Each cage of flies was supplied with adult feed (a mixture of sugar, dried chicken egg yolk and protein hydrolysate) in a small plastic cup and water *ad libitum*. Water was supplied via a length of kitchen sponge (6cm x 3cm x 0.4cm thick) one end of which was inside the cage and the other protruded from the cage into a trough of water placed under a row of six cages. With the sponge waterer and the feed cup the total resting area available inside the cage was about 200cm^2.

Waxed oviposition panels were removed each day and all eggs collected and counted. Daily counts were made of all oviposition holes in the oviposition panels as well as the number of dead males and females.

Collected eggs were placed on damp black filter paper on a piece of damp sponge all of which was placed in a lidded Petri dish as stored at 25°C. After 3 or 4 days egg hatch was assessed under a binocular microscope.

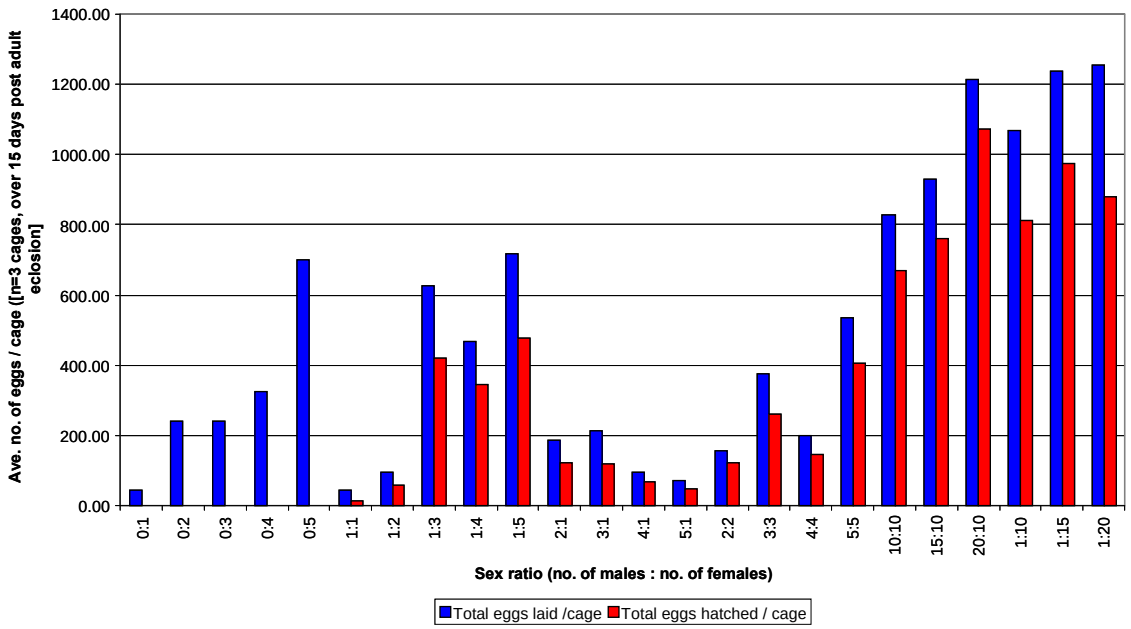

Figure 25. Effects of sex ratio and number of flies per cage on egg production and fertility of *Bacrocera oleae*.

There were three replicate cages for each ratio treatment giving a total of 72 cages. Statistical analyses need to be completed on this work.

Figure 25 shows that egg production and fertility increased, although not linearly (see also **Figure 26**) with an increase in the number of females per cage. The highest production of fertile eggs came from cages with 10, 15 or 20 females and 1, 10, 15 or 20 males. The fecundity and fertility of 10, 15 or 20 females with just one male is remarkable and warrants further investigation.

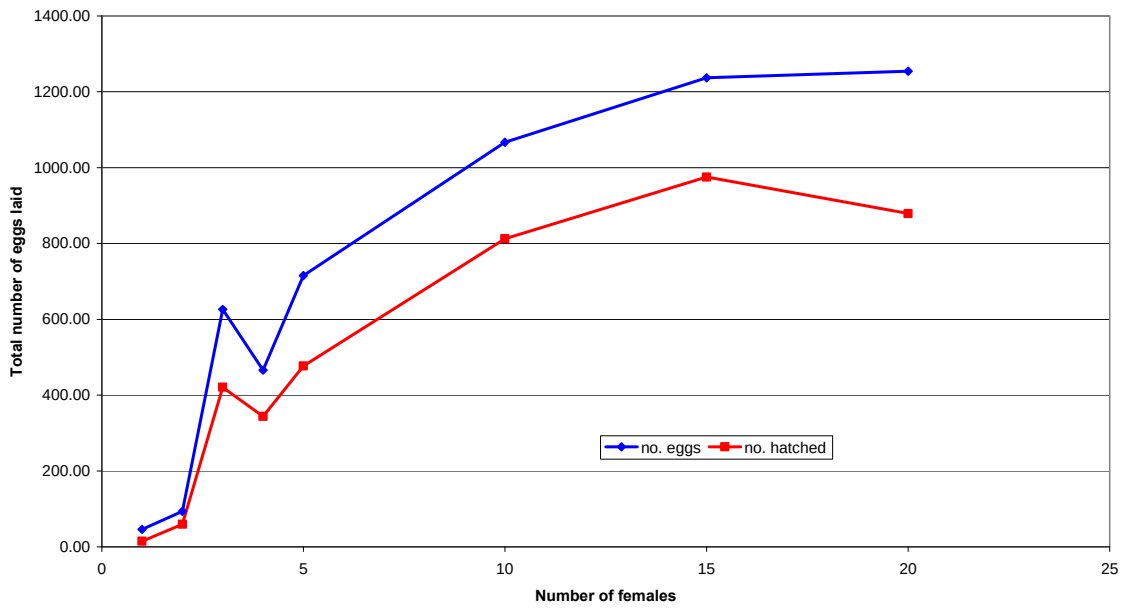

Figure 26. Average number of *Bactrocera oleae* eggs laid / cage by a number of females with just one male.

The highest production of fertile eggs was achieved at a ratio of 20 males to 10 females (sex ratio of 2:1). That is 30 insects in 116 cm^3 (1 insect in 3.9 cm^3) or on 144 cm^2 (1 insect per 4.8 cm^2) surface area. This concentration of insects per cage is slightly less restrictive than the 1 insect per 3.9 cm^2 for Mediterranean fruit fly mass-reared for SIT in Metapa, Mexico. It has been suggested that *B. oleae* should not be caged under such high adult densities because of their requirement to create their own oviposition puncture. But this experiment shows that high densities may be achievable. However, the rearing conditions in this experiment may have been inadvertently optimal for adult survival and egg production. A favourable environment depends on light quality, availability and duration, access to feed, water and space, available oviposition space, relative humidity and possibly other conditions.

When just one female was exposed to a variable number of males (**Figure 27**) the ratio of between 2 and 3 males to 1 female produced the highest number of fertile eggs per cage over the 15 days of the experiment. In this experiment the highest number of eggs laid in one day per female was 58 (of which 44 were fertile) and the second highest was 52 eggs laid (with 43 hatched) (**Table 9**). These data came from a cage with 2 males and 1 female (sex ratio of 2:1) and 3 males to 1 female (3:1) respectively. An egg production rate of about 15 to 20 eggs per

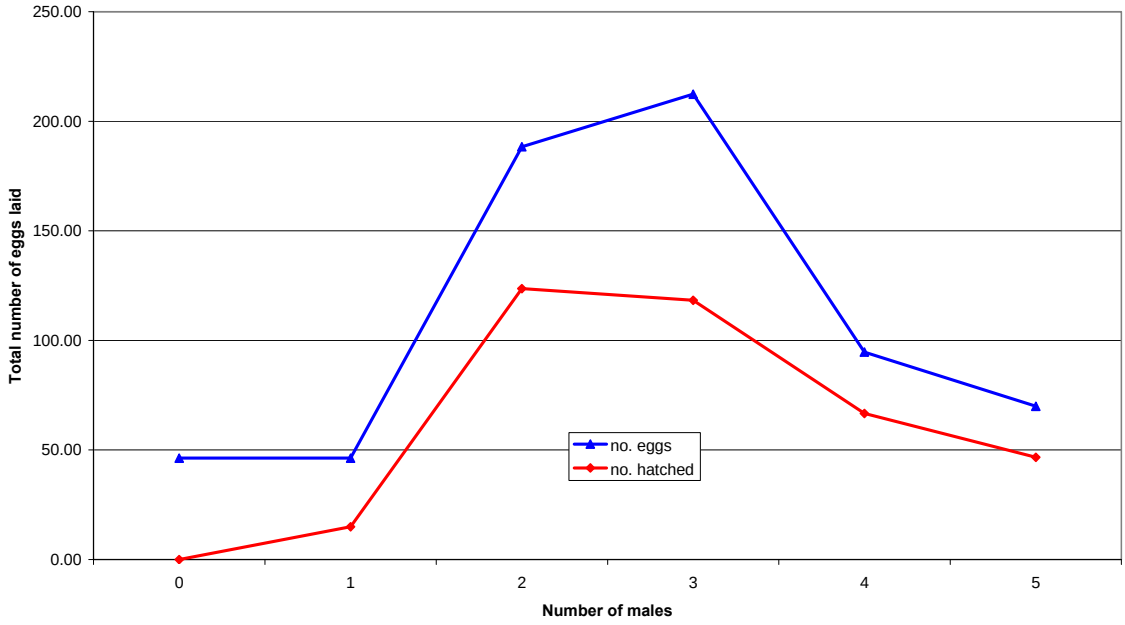

Figure 27. Average number of *Bactrocera oleae* eggs laid/cage by a single female with a variable number of males.

female per day is, at present, an acceptable level of production. A figure of over 50 might be seen as the optimum production rate and could be the target for future improvements to mass-rearing *B. oleae*.

Table 9. Productivity of female *Bacrocera oleae*.

	Eggs/live female/day	Hatched eggs/live female/day
Average	21.1	17.0
Maximum	58.0	44.0
Minimum	0.0	0.0
St Dev.	11.409	8.749

Note: These data account for female mortality during the 15 day period (post adult emergence) the experiment was run and is the average result from each cage (from a total of 72 cages).

As mentioned above, a sex ratio of about 2:1 to 3:1 seems optimal and should be studied further. Adult density needs to be tested under different loads. Also the real environmental optima for efficient mass-rearing need to be delineated.

3.3. Genetic Sexing Fruit Flies

3.3.1. Genetic, Cytological and Molecular Analyses of Genetic Sexing Strains from Two Mass-rearing Facilities

In 1994 the first genetic sexing strain (GSS) for the Mediterranean fruit fly was introduced into an operational mass-rearing facility (Petapa, Guatemala). After several years of evaluation the strain was transferred to the newly built facility in El Pino, Guatemala. Since then this facility was enlarged significantly and has today a maximum production capacity of ca. 2 500 million males per week.

The strain used was VIENNA 4 with its autosomal translocation breakpoint at 57A on the right arm of chromosome 5 (**Figure 28**). This GSS is based on the selectable markers white pupae (*wp*) and temperature sensitive lethal (*tsl*). The position of the autosomal translocation breakpoint is closer to *wp* and *tsl* than in older GSS like 30C (T(Y;5)101) and, therefore, the genetic stability with respect to Type-1 male recombination is improved. Type-1 recombination occurs in males prior to meiosis and is a homologous recombination event between the translocated and the free autosome in the chromosomal area between the translocation breakpoint and the selectable markers. If it occurs it produces in case of VIENNA 4 recombinant females with a wild type phenotype (wp^+ tsl^+) that cannot be

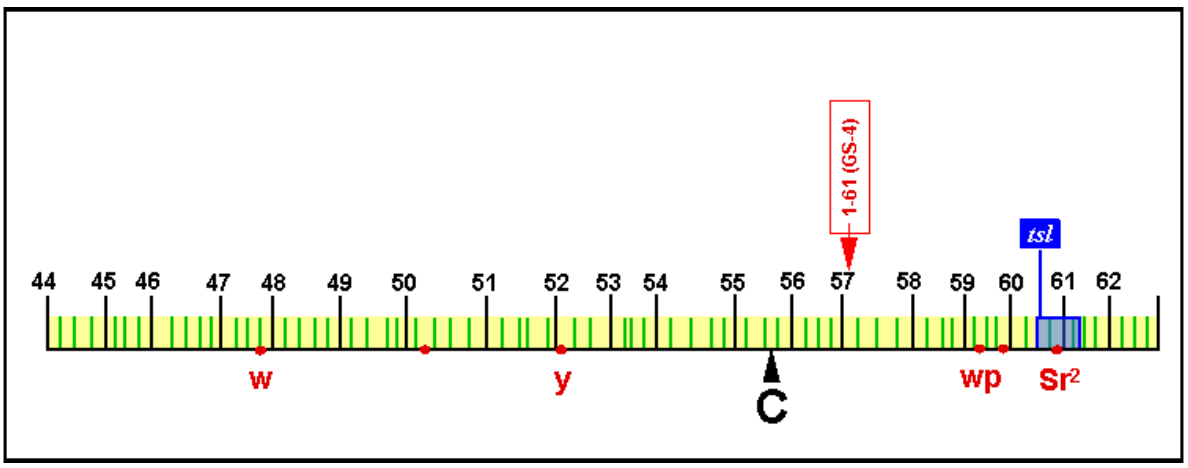

Figure 28. Schematic representation of the polytene chromosome 5 isolated from trichogen cells. The autosomal translocation breakpoint of the GSS VIENNA 4 (= 1-61) is shown. In addition the location of the 2 selectable markers *wp* and *tsl* are indicated together with other phenotypic markers (w (white), y (yellow body) and Sr^2 (Sergeant 2)).

removed from the males during the heat treatment to kill the females. However, they can be removed in the clean stream of the Filter Rearing System due to their pupal phenotype. Furthermore, these recombinant females accumulate in the colony because they have a selective advantage over their non-recombinant sisters. The male recombination frequency in VIENNA 4 is reduced to 0.014% per generation (calculated after excluding adjacent-1 offspring) as compared to 30C (T(Y;5)101) where male recombination occurs at a frequency of 0.111% per generation. In VIENNA 4 the translocation breakpoint on the Y chromosome is located in the middle of the long arm, i.e. in an area that is occupied by repetitive DNA sequences. As a consequence, the two resulting Y-chromosomal translocation fragments share

a high level of similarity at the DNA level. This leads to the occurrence of Type-2 recombination, i.e. the illegitimate exchange between adjacent chromosomal elements based on DNA sequence homology. The consequence is the reversion of the Y-autosome translocation back to a free Y chromosome. The resulting recombinant males are fully fertile and accumulate very rapidly in the mass-rearing colony. Fortunately Type-2 recombination occurs at a much lower frequency than Type-1 recombination, i.e. it can be estimated that the frequency is in the order of 10^{-5} % per generation. Secondly, these males are temperature sensitive because they are homozygous for the *tsl* mutation and are therefore killed together with the females.

Before the introduction of VIENNA 4, which is based on genetic material from Egypt, into the Petapa facility it was discussed whether the strain would be sufficiently compatible with the wild flies in Guatemala. As a safety precaution it was therefore decided to outcross VIENNA 4 with a relatively recently colonized wild type strain from Guatemala (Toliman strain). The outcrossing scheme involved 3 consecutive crosses with Toliman flies. The resulting strain was named VIENNA 42/Tol. It is important to note that at least one of these crosses was done with Toliman females, i.e. as the mitochondrial DNA (mtDNA) is inherited only through the female the mtDNA haplotype of the Toliman strain was introduced into the GSS for Guatemala. The mtDNA was shown to be an excellent marker to study population structures and to identify strains (for example see Activities Report 2001). The differentiation of different haplotypes is achieved by PCR amplification of certain regions of the mitochondrial genome followed by the digestion with an appropriate restriction enzyme that is known to be polymorphic in different strains/populations (**Figure 29**).

So far 4 enzymes have been used to detect polymorphisms: EcoRV, XbaI, MnlI and HaeIII (see Activities Report 2001). The different mtDNA haplotypes are described as follows: The presence of a restriction site in the respective region of the mitochondrial genome is designated by the letter A, the absence with a B. Only in case of MnlI with several restriction sites in the respective DNA fragment the letters used in the description of the haplotype refer to different patters rather than to the presence/absence of a single site. The order of the different enzymes is always as given above. The mtDNA haplotype of the Toliman strain is AAAB, i.e. the restriction sites for the enzymes EcoRV and XbaI are present, MnlI produces a particular pattern designated as :A" and the site for HaeIII is absent in the particular part of the mitochondrial genome. This pattern is very typical for Central America (**Figure 30**). In particular the HaeIII polymorphism is very useful to distinguish the released

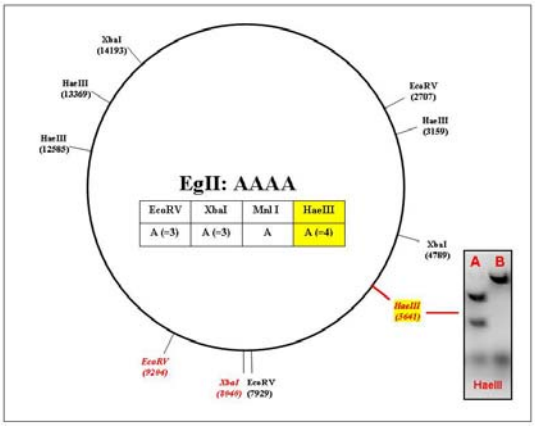

Figure 29. A map of the mitochondrial genome of the Mediterranean fruit fly Three of the four polymorphic restriction enzyme markers used for strain identification are indicated. As an example a AAAA haplotype is shown as it is found in the wild type strain EgII. In the insert a gel electrophoresis of the two variants of the HaeIII site are shown.

strain from most wild type populations as the "A pattern" is restricted primarily to Egypt and Greece. Rare cases are found in South Africa and there the entire set of all 4 enzymes is required to distinguish the released strain from all individuals of the wild population. The Egypt haplotype AAAA was so far not reported outside of Greece and Egypt (unpublished results suggest that very rarely this haplotype was detected in Central America).

In 1999/2000 the GSS in Guatemala was upgraded to an improved version. The GSS used was VIENNA 7 with a translocation breakpoint at 58B (**Figure 31**) on the right arm of chromosome 5. The advantages of this strain are:

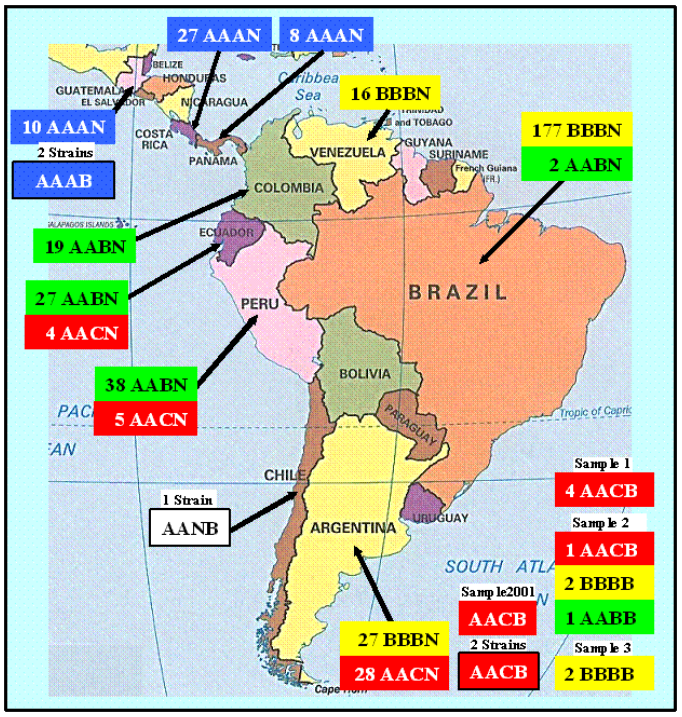

Figure 30. Distribution of different mtDNA haplotypes. In this map data from Bruce McPheron and our data are combined. The prevailing haplotype in Central America is AAAB.

a) The translocation breakpoint is closer to the selectable markers white pupae (*wp*) and temperature sensitive lethal (*tsl*) than in VIENNA 4 and therefore the male recombination frequency is reduced even further (0.011% per generation). As a consequence, the strain is very stable with respect to Type-1 recombination.

b) The translocation breakpoint on the Y chromosome is located close to the centromere, i.e. the two resulting Y-chromosomal translocation fragment are relatively dissimilar which reduces the frequency of Type-2 male recombination.

c) The triplication type adjacent-1 offspring produced by the segregation of this translocation are female and only very few survive to the adult stage (for details see below). Therefore these genetically unbalanced individuals do not affect negatively QC parameters like adult emergence, percent deformed/crippled flies, percent fliers and the mating efficiency as it is the case for earlier GSS, e.g. VIENNA 4.

The introduction of VIENNA 7 was accomplished by three consecutive crosses with females derived from the existing VIENNA 42/Tol strain. Thereby the VIENNA 7 translocation is introduced but the Toliman genetic background, the Toliman mtDNA haplotype AAAB and the selectable markers *wp* and *tsl* are maintained in the resulting strain.

In 2001 and 2002 this strain was also introduced into the newly renovated CDFA facility on Hawaii.

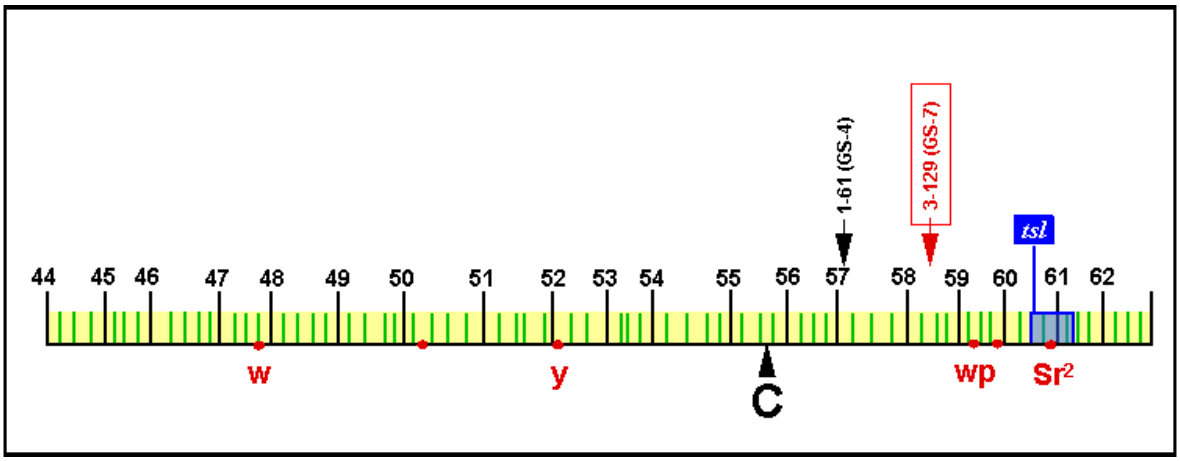

Figure 31. Schematic representation of the polytene chromosome 5 isolated from trichogen cells. The autosomal translocation breakpoint of the GSS VIENNA 7 (= 3-179) is shown. In addition the location of the 2 selectable markers *wp* and *tsl* are indicated together with other phenotypic markers (w (white), y (yellow body) and Sr^2 (Sergeant 2)).

In the past genetic stability of the GSS was of primary concern. This was the consequence of very negative experiences with the first generation of sexing strains (e.g. with the GSS 30C (TY;5)101) that resulted in reservations to implement GSS in operational programmes. After the stability issue was solved and the stable GSS were proven to function successfully in SIT programmes, the attempt was made to improve the productivity of GSS. Reduced productivity of GSS is the consequence of adjacent-1 segregation during male meiosis. Due to this effect most GSS produce 50% genetically balanced and 50% genetically unbalanced offspring. The unbalanced offspring either die during the rearing process or represent flies with very poor quality. The occurrence of adjacent-1 genotypes is the consequence of the presence and, potentially, the structure of the Y-autosome translocation in the GSS males. Y-autosome translocations are induced through radiation and the structure of the translocations generated in this progress cannot be predicted. The only way to find optimal translocations is by analysing as many different translocations as possible. With respect to productivity it was known that the old translocation 30C (TY;5)101) showed very good results. However, it was impossible to use this strain because of its very low genetic stability which is the result of the fact that the translocation breakpoint is located at 52B on the left arm of chromosome 5, i.e. far away from the selectable markers *wp* and *tsl* (**Figure 32**). It was therefore required to stabilize this GSS. This was achieved by induction of an inversion on chromosome 5, D53 (**Figure 32**). This pericentric inversion covers the chromosomal region including the 30C (TY;5)101) translocation breakpoint and *wp*. Heterozygous pericentric inversions do not reduce the recombination frequency as such but rather render most recombinants inviable, i.e. the presence of an inversion in a heterozygous condition leads to a certain level of sterility. However, as the recombination frequency in males, as compared to females, is very low the resulting sterility is negligible. A new strain, VIENNA 8, was constructed by combining the inversion D53 carrying the selectable markers *wp* and *tsl* with the Y-autosome translocation 30C (TY;5)101). VIENNA 8 shows a ca 30% higher productivity (**Figure 33**) which is most likely the consequence of the fact that in these males the frequency of adjacent-1 segregation is reduced significantly to the benefit of alternate segregation, i.e. more genetically balanced

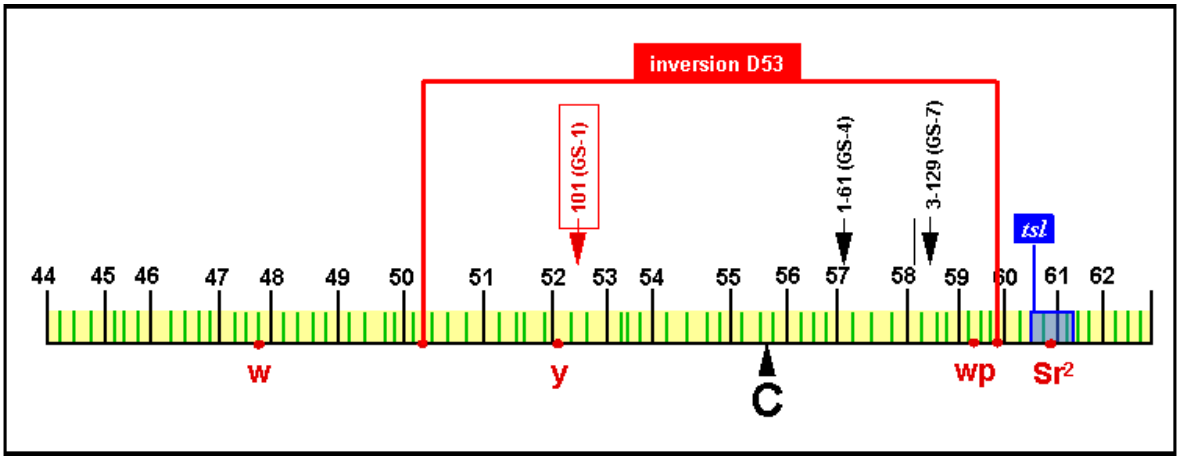

Figure 32. Schematic representation of the polytene chromosome 5 isolated from trichogen cells. The autosomal translocation breakpoint of the GSS VIENNA 8 (= 101) is shown. In addition the breakpoints of the inversion D53 are shown. The location of the 2 selectable markers *wp* and *tsl* are indicated together with other phenotypic markers (w (white), y (yellow body) and Sr^2 (Sergeant 2)).

sperm is produced. Furthermore, the stability of the strain with respect to Type-1 recombination is comparable to VIENNA 4 (0.014% per generation). Like in VIENNA 7 the translocation breakpoint on the Y chromosome is located close to the centromere, i.e. the two translocation fragments are relatively dissimilar and as a consequence Type-2 recombination is reduced. In addition, during the translocation process the Y chromosome in the translocation 30C (TY;5)101) was severely modified. At least one deletion and one inversion must have occurred which may be the reason for the special segregation behaviour of this translocation. Furthermore, these rearrangements resulted in a very distinct, easily recognizable appearance. During the construction of the strain it was ensured that it carries the AAAA mtDNA haplotype.

Early in 2003 VIENNA 8 was transferred to Guatemala for evaluation.

In the course of evaluations to determine the suitability of VIENNA 8 for operational programmes and to verify its advantages, it was re-analysed in Seibersdorf. For this purpose the strain was compared to material sent from the Guatemala mass rearing strain, i.e. VIENNA 7/Tol. In this test the overall productivity of both strains was measured. The surprising result was that VIENNA 7/Tol showed the same high level of productivity as VIENNA 8.

In 2008/2009 these tests were repeated with fresh material from the Guatemala mass rearing strain. Eggs were collected for 5 h, incubated at 25°C for 24 h and then treated for 24 h with different temperatures between 31°C and 35°C. The egg hatch, pupal recovery and the number of emerged adult males and females was determined. The standard temperature to kill the homozygous *tsl* females is 34°C. The temperature treatment was applied because it is known that adjacent-1 individuals are more temperature sensitive than the balanced genotypes, especially when the *tsl* is present in the strain. Therefore, the response to different temperatures allows conclusions about the abundance and the lethal phase of the adjacent-1 individuals in different strains. These characteristics are reproducible features of each individual strain, more precisely of each individual Y-autosome translocation and its resulting

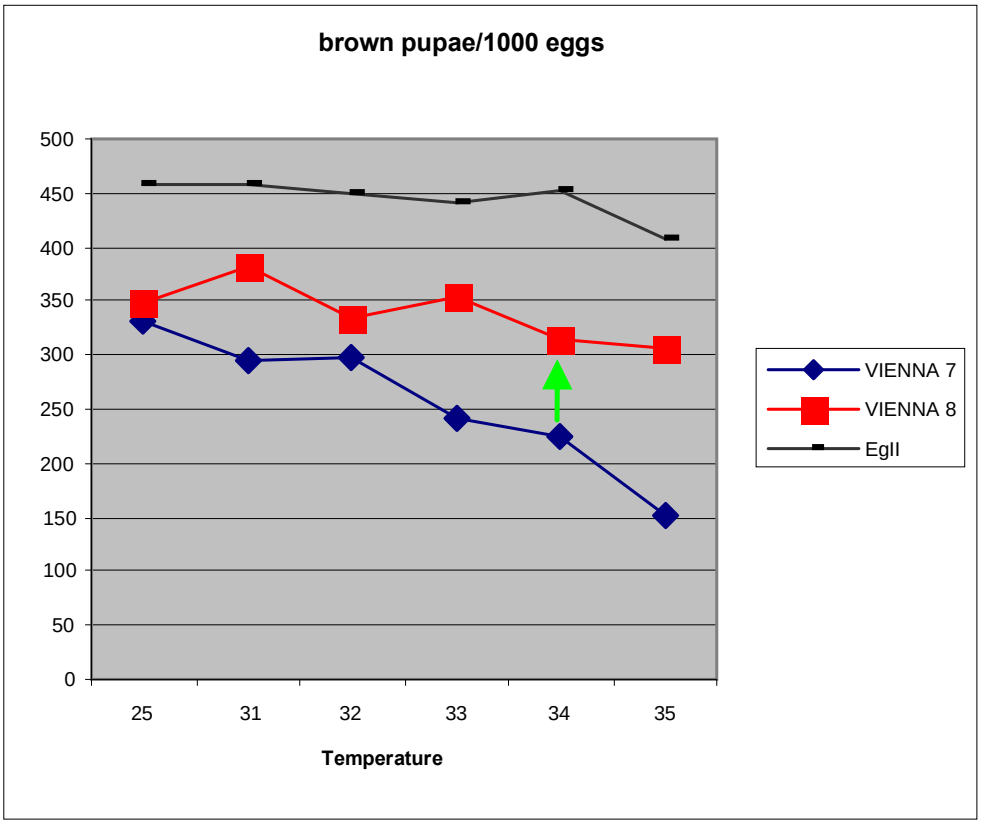

Figure 33. The productivity, expressed as brown (male) pupae per 1000 eggs, is compared between VIENNA 7, VIENNA 8 and the wild type strain EgII. The productivity was determined at different temperatures applied for 24 h after collecting the eggs for 5 h and after maintaining the eggs for 24 h at 25°C. At the discriminating temperature of 34°C (all females are killed) the productivity of VIENNA 8 is ca 30% higher than with VIENNA 7.

segregation behaviour during male meiosis, and can be used to distinguish different strains. For the comparison of VIENNA 7 and VIENNA 8 the most informative stage is the pupal stage because the egg hatch data are strongly affected by the gradual disappearance of the females with increasing temperature, i.e. any difference that is present is less obvious. VIENNA 7 is characterized by a significant temperature dependent reduction in pupal recovery which is linked to the viability of the adjacent-1 genotypes (**Figure 34**). In this particular strain the temperature dependent lethal phase of the adjacent-1 individuals (triplication type, **Figure 35**) is delayed due to the fact that these female adjacent-1 individuals, emerging from brown pupae, carry:

a) only a relatively short duplication (chromosomal region 58B to 62B, distal region of the right arm) and

b) a tsl^{+} allele in addition to the normal two doses of the *tsl*.

This becomes obvious when the *tsl* is removed, i.e. in a strain carrying a *w wp* chromosome instead of the *wp tsl* chromosome, the temperature dependent lethality disappears (VIENNA 7 versus VIENNA 7/*w wp*, **Figure 34**). The structure of the translocation in VIENNA 8 is very

different. The triplication type adjacent-1 individuals carry a duplication of the chromosomal segment between 44A and 52B (distal region of the left arm) and are homozygous for the *tsl*, i.e. they do not carry an additional tsl^+ allele and are with respect to the *tsl* like the normal females and die early during embryo development. As a consequence pupal recovery is not affected by temperature (**Figure 34**).

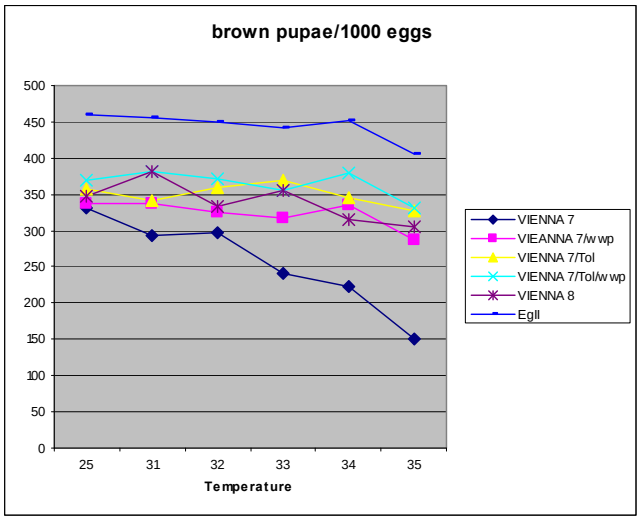

Figure 34. The productivity, expressed as brown (male) pupae per 1000 eggs, is compared between VIENNA 7 from Seibersdorf, VIENNA 7 from Seibersdorf outcrossed with *w wp* females, VIENNA 7/Tol from Guatemala, VIENNA 7/Tol from Guatemala outcrossed with w wp females and VIENNA 8 from Seibersdorf (control: wild type strain EgII). The productivity was determined at different temperatures applied for 24h after collecting the eggs for 5h and after maintaining the eggs for 24h at 25°C. The productivity profile of the VIENNA 7/Tol strains is identical to VIENNA 8 but distinctly different from VIENNA 7.

A comparison between VIENNA 8 from Seibersdorf and VIENNA 7/Tol shows clearly that these two strain are indistinguishable (**Figure 34**). Furthermore, males of the VIENNA 7/Tol strains were crossed twice with *w wp* females. The results in **Figure 34** show that the high pupal recovery is maintained. Therefore, it can be concluded that the high recovery in VIENNA 7/Tol from Guatemala is the consequence of the Y-autosome translocation, i.e. its segregation behaviour during male meiosis, and is not due to unknown factors in the genetic background that may have been introduced into the strain via selection during the many years of mass rearing in Guatemala. In addition to the earlier evaluation the following additional analyses were conducted:

a) mtDNA haplotype: 37 individual of VIENNA 7/Tol from Guatemala were analysed and all of them showed the AAAA mtDNA haplotype and not the AAAB mtDNA haplotype as expected.

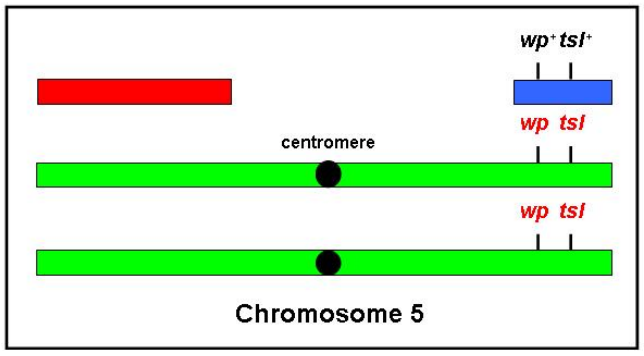

Figure 35. Schematic representation of the structure of the triplication type adjacent-1 females in VIENNA 7 (blue) and VIENNA 8 (green).

b) Cytology: VIENNA 7/Tol was analysed with respect to the location of the autosomal translocation breakpoint (in collaboration with Antigone Zacharopoulou, University of Patras, Greece). For this the isolation of the trichogen cells responsible for forming the male-specific SOF bristles is required (two cell per male head). Consequently

only very few individuals could be analysed but the results were very clear. The translocation breakpoint is at 52B on the left arm of chromosome 5, i.e. it is identical to VIENNA 8 and so far away from the translocation breakpoint in VIENNA 7 (58B, right arm of chromosome 5) that it cannot be confused. However, none of these few individuals analysed showed the inversion that is present in VIENNA 8.

In 2009 also a sample of dead flies was obtained from the Hawaii mass rearing facility. Here it was only possible to determine the mtDNA haplotype. 65 individuals showed the AAAA mtDNA haplotype and only 15 the expected AAAB mtDNA haplotype.

In conclusion the results are very puzzling. Neither of the two strains in Guatemala and Hawaii shows the expected characteristics. Both strains are mixtures of components characteristic for VIENNA 7 and VIENNA 8 (**Table 10**). The mass rearing strain from Guatemala shows all the features of VIENNA 8, except the inversion and the Hawaii mass rearing strains shows a mixture of mtDNA haplotypes. Currently there is no good explanation how it was possible that two of the VIENNA 8 features, translocation and mtDNA haplotype, were introduced into the Guatemala mass rearing without introducing the third feature, the inversion, at the same time. And there is no good explanation how it could happen that the Hawaii strain shows a mixture of haplotypes while the parental strain in Guatemala apparently does not.

Table 10. The data obtained with samples from two mass-rearing strains from Guatemala and Hawaii, respectively, were compared to older data for VIENNA 7/Tol maintained in Seibersdorf and with the data for VIENNA 8. Green: identical to VIENNA 7/Tol from Seibersdorf, yellow: identical to VIENNA 8.

	VIENNA 7/Tol (Seibersdorf)	VIENNA 8 (Seibersdorf)	VIENNA 7/Tol Guatemala	VIENNA 7/Tol Hawaii
Translocation breakpoint	**57A**	**52B**	**52B**	**Not yet determined**
Viability	**ca 50%**	**ca 75%**	**ca 75%**	**Not yet determined**
Inversion	**No**	**Yes**	**No**	**Not yet determined**
mtDNA haplotype	**AAAB**	**AAAA**	**AAAA**	**19% AAAB** **81% AAAA**

For the SIT programme in California, currently receiving flies from Guatemala and Hawaii, it would be very important to use the mtDNA marker AAAA as a way to determine the mating status of trapped female flies. This was one of the arguments to suggest changing the strains in these facilities to VIENNA 8 assuming at that time that the current strains contain a mtDNA haplotype that is not informative for this analysis. The new results show that at least the males from Guatemala would allow a mating status analysis. However, due to the mixture present in the males from Hawaii this is made impossible.

It is planned to obtain live material from the Hawaii strain to complete the analysis, i.e. the analysis of the productivity and the cytology. Furthermore, a new VIENNA 8 strain is being prepared in Guatemala by outcrossing with wild material. Tests are planned to verify that the correct components are included in this strain.

3.4. Mosquito Rearing

3.4.1. Mass-Rearing Tray Design

The first thermoformed plastic prototype tray described in Activities Report 2008 has been produced and tested (**Figure 36, left**). The larval rearing experiments were conducted with *Anopheles arabiensis* Dongola strain using the IAEA larval diet (see composition below). The trays were tested with 3 500 first instar larvae (L1) per tray, which is equivalent to 1 L1 per 1.7 cm^2. This density was chosen based on the results obtained in small-scale experiments (Petri dishes) and from preliminary work on these trays. These conditions offered a good development environment for the immature stages. However, with time, in the old tray prototype the food that was offered daily was accumulating in the deeper central part of the tray (2.5 cm depth) and becoming inaccessible to the young larvae, which feed mainly on the water surface level.

The second prototype tray design (**Figure 36, right**) has the same external dimensions of 600 x 1 000 mm and a height of 3 cm. The shorter sides of the tray have slopes of 15° to facilitate the discharge of the water by raising one side by approximately 25 cm. The bottom of the tray is flat ensuring a constant water depth over the entire tray, which should prevent or limit the accumulation of food in the central part. Food accessibility will be higher, reducing mortality and consequently cost. Furthermore, the central ridge has been divided into two parts to promote a better food and water circulation inside the tray. In addition, the purpose of the central ridge is to give the tray increased structural stability and to increase the resting space for the larvae. An overflow system has been created by drilling one or more holes in the ridge. In this way, a stack of trays can be filled from top to bottom according to a cascade system.

Figure. 36. The first prototype tray design (left) and the second prototype version (right). Top left insert – overflow system, bottom right insert – central ridge.

The trays will be tested at different water temperatures and feeding schedules in order to define the optimal rearing conditions with the aim to improve the synchrony of pupation and to reduce mortality.

3.4.2. Larval Tray Holding Rack

A stainless steel rack prototype for holding the larval trays has been developed (**Figure 37, left**). The rolling stainless steel structure is 190 cm high and able to hold 50 trays. Depending on the rearing larval density used in each tray, this rack could lead to the production of around 200 000 mosquito pupae. The actual model includes an endless screw jack fixed on the rack, which allows all the trays to be lifted simultaneously by moving an inner frame connected to each tray. This lifting system moves inside the main structure and will not require a movement of the main frame of the rack (**Figure 37, right**). In order to collect the mosquito pupae at the end of the pupal period, new modifications for the draining side were required and are now under construction. These modifications consist of a plastic undulating panel to gently drive the pupae towards the bottom of the rack where a netted basket for pupae collection will be placed.

Figure 37. Mosquito stainless steel rack that can stack 50 larval holding trays showing the endless screw jack for the tilting procedures (left). The rack tilted in the draining position (right).

During preliminary experiments, trays were stacked inside the rack and showed adequate air flow and light requirements for larval rearing. The differences in temperature of the water in the trays located at different levels in the rack will be further investigated.

3.4.3. Larval Pupa Separator

Unlike for other mass-reared pest insects such as fruit flies, tsetse flies, etc., rearing procedures for mosquitoes require a water environment for the immature stages. Considering that synchronized pupation is not achievable even under optimal conditions, it would be necessary to separate the larvae from the pupae so that larvae can be returned to the production line to complete development and the pupae prepared for irradiation, transport and release. In a mass-rearing process, the pupae must be completely removed from the larvae to prevent emergence of excessive numbers of adults into the production facility. However, a small percentage of larvae in the pupal harvest can be acceptable. The most efficient pupae-larvae separation method used for Anophelinae species exploits the typical difference in buoyancy between pupae and larvae in the absence of movement (active behaviour). This method allows a good separation especially when the natural swimming activity is disturbed by swirling the water (vortex) or reduced by adding chilled water. After identifying the most optimal temperature (10°C) allowing separation with a minimal stress for *An. arabiensis*, we tested whether cold and swirling effect could be combined in a synergistic way. A first prototype has been designed, constructed and tested to evaluate the separation efficacy and the stress induced by the process (**Figure 38**).

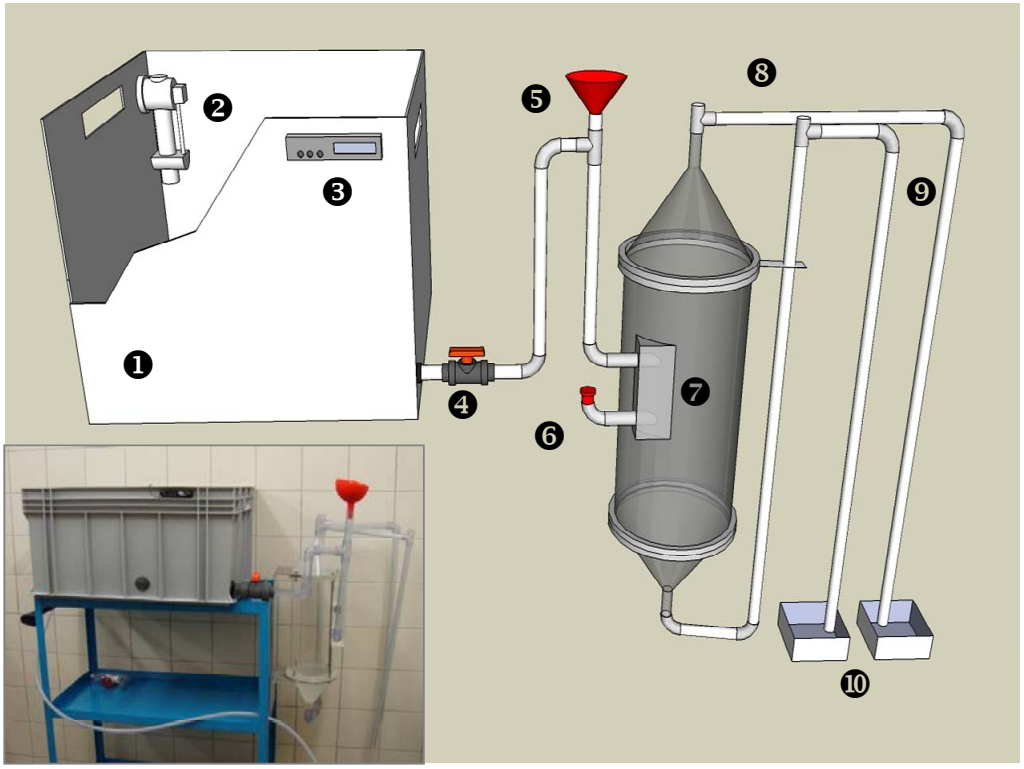

Figure 38. Larvae/pupae separator. Diagram and picture of the separation system tested in the experiments. 1 - Reservoir water tank. 2 - Floating valve. 3 - Digital thermometer with submerged water probe. 4 - Flow valve. 5 - "Reverse U-shaped" pipe line and vertical injection pipe for larvae-pupae mixture. 6 - Double inlet connector. 7 - Separation chamber. 8 - Larvae and pupae collector pipes. 9 - Larvae and pupae conveyor pipes. 10 - Larvae and pupae collection boxes.

Survival and metamorphosis rates (**Figure 39A,B**) were not affected by the passage of the immature stages through the separator. The separation efficacy of the system processing batches of 1000 larvae and pupae (LP) (ratio 1:1) is shown in **Figure 40**. Using the system with a water flow of 4 liter per minute and a temperature of 15°C, 98.5% of the pupae and 97.5% of the larvae processed were separated, which was also confirmed in larger-scale separation tests using 30 000 LP mixtures (1:1). The separation effects of the two classical methods (vortex and cold water) appear to work synergistically in this device and permit an efficient separation. The separation time in all trials was below 2 minutes, confirming the possibility of separating 1 million LP mixtures within 2 hours.

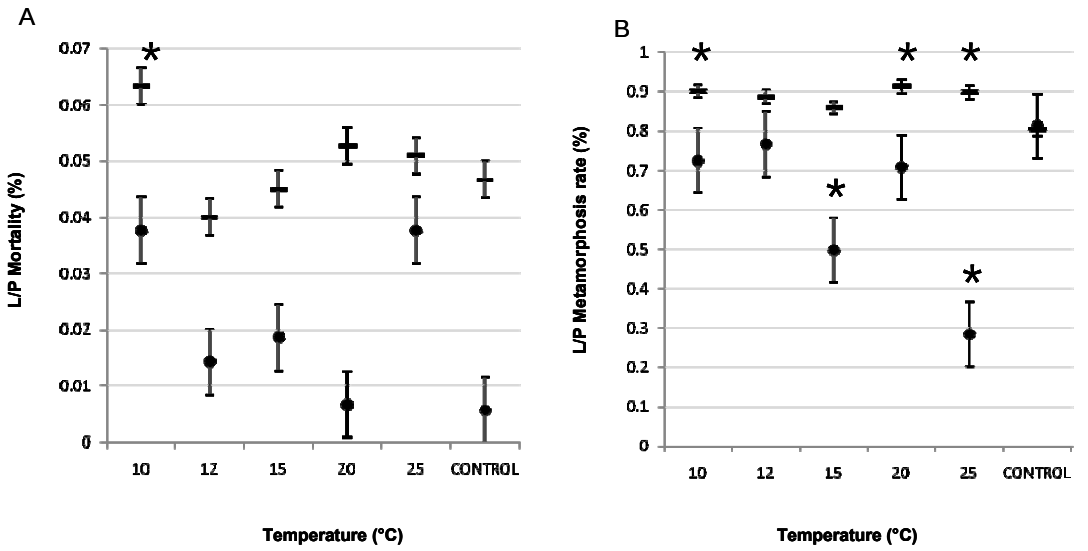

Figure 39. Relationship of temperature to (A) mortality and (B) metamorphosis rates (e.g. larvae developing in pupae and pupae developing into adults) in samples of larvae (●) and pupae (─) that had passed through the separator at various temperatures. Vertical bars represent the standard error of the mean. Asterisks mark groups whose odds ratios are significantly different with reference to the control. The larger of the two overlapping standard error bars in panel B for the control value is that of the larval data.

3.4.4. Mass Production Cage and Oviposition Behaviour Studies

Large-scale production of mosquitoes is a key factor for a successful SIT programme. An efficient adult mass-production cage must contain appropriate features for adult resting, feeding, mating and oviposition activities.

In order to maximize egg collections, tests were conducted to determine the physical characteristics of oviposition sites for caged *An. arabiensis*. Effects of texture, shade, height and shape on female oviposition behaviour were investigated. Results indicated a strong preference for oviposition on humid substrates over free-standing water. The shade and texture of the cup walls also influenced site choice; black rough inner vertical walls of the cup resulted in the largest number of eggs. Oviposition sites with square shape were preferred by females rather than circular cups, and in the square cups, over 60% of the eggs were laid in the corners (**Figure 41A**). Height also affected oviposition: *An. arabiensis* significantly favoured sites on the floor even though some oviposition occurred at higher sites (**Figure 41B**).

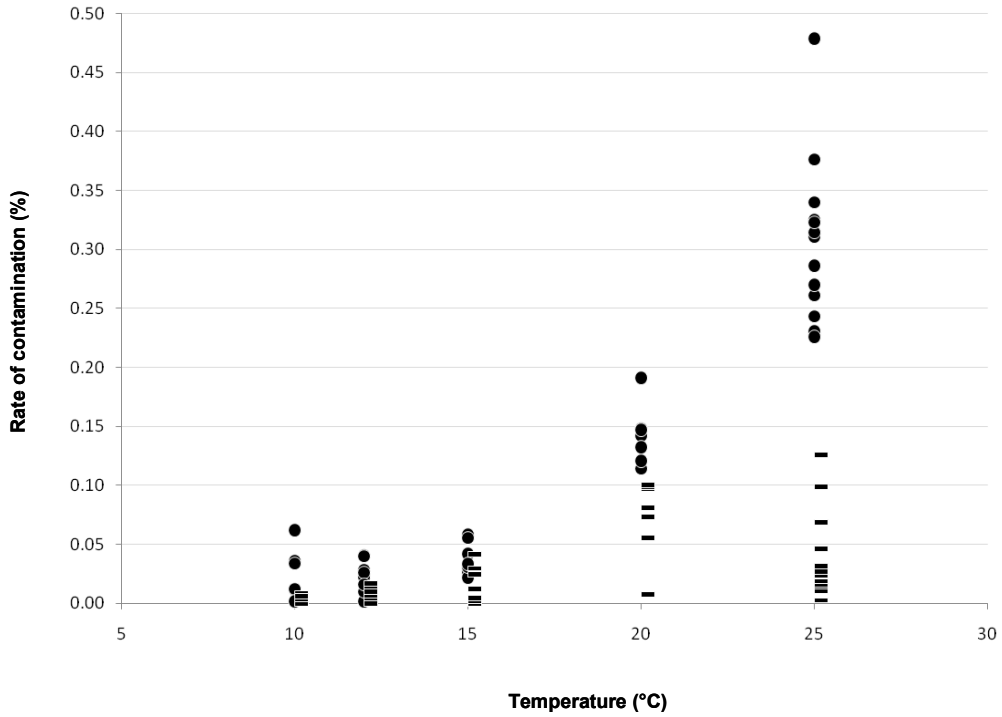

Figure 40. Relationship of temperature to rate of contamination of the pupal stream by larvae (●) and the larval stream by pupae (—). (Experiments carried out with 1000 larvae/pupae mixtures).

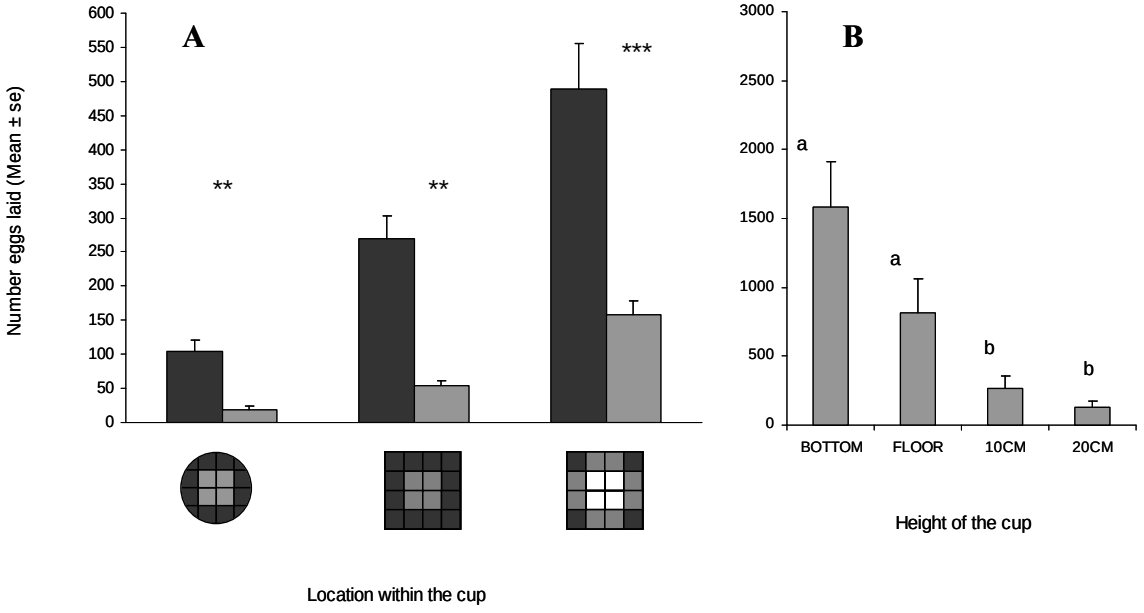

Figure 41. A - Mean number (± SE) of eggs laid by females according to different locations inside the oviposition cups ($P < 0.05$; *** $P < 0.001$). **B -** Mean number (± SE) of eggs laid by females as a function of the height of the oviposition cup (different letters indicate significant difference).

Based on this study and from available literature, we determined the characteristics of an artificial oviposition site that included all the characteristics that yield large numbers of eggs required for a mass production of this species, and these were integrated in the design for the adult mass production cage (**Figure 42**).

The blood feeding system used in the first version of the mass production cage had a heating metal mesh connected to a collagen membrane. Studies conducted in small cages showed that both the membrane and the heating system created poor attraction to females. In addition, the few females attracted to the system had difficulties with engorging themselves. A new blood feeding system was developed and tested based on the Hemotek insect feeding machine (Hemotek® PS6A/220) and modified with an enlarged aluminium blood plate, covered by a collagen membrane kept in position by a plastic ring. The blood is replenished using disposable luer-lock syringes (50ml capacity) (**Figure 43**).

A new emergence tube for the pupae has been designed with a larger opening allowing easier "take-off" of the adults. The tube will be fixed at a distance of more than 30 cm measured from the bottom of the cage. Oviposition plates are positioned on the floor of the cage (**Figure 42**). An aspiration system was added to the bottom of the cage to allow collection of the dead adults, which will accumulate under a stainless steel mesh net. This metal mesh placed at 5 cm from the bottom will be used as floor level for the oviposition plate.

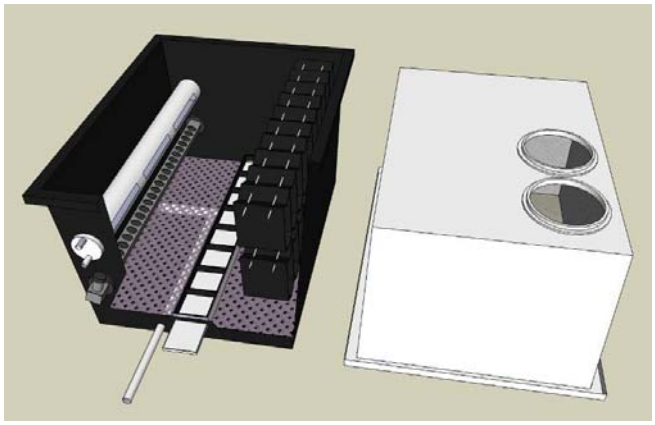

Figure 42. Adult mass production cage design. The bottom part of the cage (black) is displayed in section in order to show the eclosion tube, the sugar feeder, the eggs collection plate, the resting sites and the aspiration pipe overlaid by the meshed bottom of the cage. In the upper part of the cage (white) are shown the opening for the blood feeders.

Figure 43. Blood feeder plate connected to the Hemotek heater and the syringe used for blood replenishment. On the right is shown the membrane employed and the plastic ring to fasten it to the plate.

3.4.5. New Diet for Anopheles arabiensis *and* Aedes albopictus *Mass-rearing*

Mosquitoes have been reared in the past using numerous larval diets that were usually mixtures of commercially available products. Their usefulness has generally been determined by assessing production parameters of mosquitoes in the colony. However, male mosquitoes that will be used for sterile insect technique programmes must have a good mating competitiveness. Given that much of the potential adult performance is influenced by the diet provided to the larvae, it is important to select diets, which not only allow high productivity in

the colony, but also provide the necessary components for adequate adult performance in the field including longevity and flight.

Several powdered larval diets were tested for their capacity to support *Anopheles* mosquito culture in trays. In addition to nutritional value, we considered cost and global availability. We carried out a stepwise selection process in which the main components were selected, followed by determination of the optimal proportions of each using a mixture design. The initial outcome variables considered were survival up to the pupa and adult stages, pupation, emergence times, and male and female wing length. Because it has been demonstrated that essential polyunsaturated fatty acids (PUFAs) are necessary for flight and mating competitiveness, a subset of the diets and mosquitoes cultured on them were analyzed for their composition of PUFAs. Effect of additives including algae and vitamin mix was also determined.

Among 10 different potential single components, only 3 ingredients (bovine liver powder, tuna meal and squid liver powder) were selected for the bulk of the diet as the initial outcome variables were significantly better with these ingredients. Using the mixture design with these 3 components, a synergetic effect was observed for bovine liver powder and tuna meal mixture, which significantly decreased the duration of development and increased the survival of the immature stages. The squid liver powder was discarded because of its antagonistic effect (**Figure 44**).

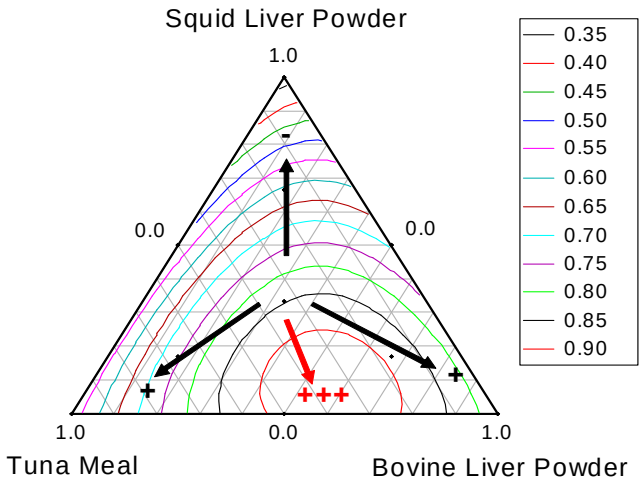

Figure 44. Mixture Contour Plot of the survival rate (1st instar larva to pupal stage) of *Anopheles arabiensis*. The different colour lines inside the triangle indicate the survival level according to the legend.

Addition of vitamin mix (essentially ascorbic acid) to bovine liver powder and tuna meal mixture increased the efficacy of the mixture and brought better stability to store the diet. This new diet will progressively replace the Koi Fish Food, currently used in our laboratory for routine purposes, as the colony performed better on this new diet for the parameters considered (immature survival, developmental time and wing length).

The final diet, which was developed using small-scale Petri dishes, showed also encouraging results for larger scale rearing. Furthermore, the diet is composed of widely available components which are cheap: one kg of the mixture (around 20 €) allows to culture one million mosquitoes. Using this new diet, *An. arabiensis* showed good flight ability and good longevity, and was easily reared in the laboratory for several generations.

Finally, the diet developed for *An. arabiensis* was tested on *Ae. albopictus*. For both species, survival rate from first instar larvae until the adult stage decreased with increasing concentrations, but *An. arabiensis* showed a greater sensitivity than *Ae. albopictus*. For *An. arabiensis,* the highest survival rate of 0.71 ± 0.03 was obtained with a diet concentration at 1% (1g diet per 100 ml water). Survival decreased drastically with higher concentrations confirming that *An. arabiensis* is a "clean water" mosquito. Almost all *Ae. albopictus* larvae survived with food concentrations between 1 and 2% and showed a survival of more than 0.70 with a food concentration of 4% (**Figure 45**).

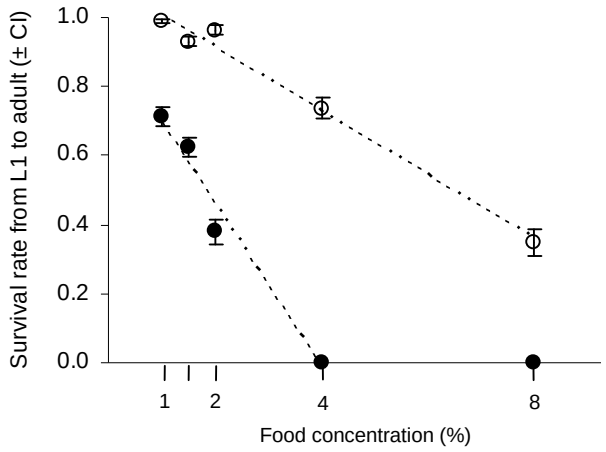

Figure 45. Mean survival rate (± CI) from 1st instar larvae to adult emergence, for *Aedes albopictus* (white circles) and *Anopheles arabiensis* (black circles) for the different amounts of diet.

For *Ae. albopictus,* adult body size, as measured by wing length measurements, increased with the amount of diet. The differences between female and male body size became more obvious when the concentration of the diet was higher than 2%. For this species, sex separation of pupae can be done using the difference in size and the larger the difference between males and females, the better the sex separation (**Figure 46**).

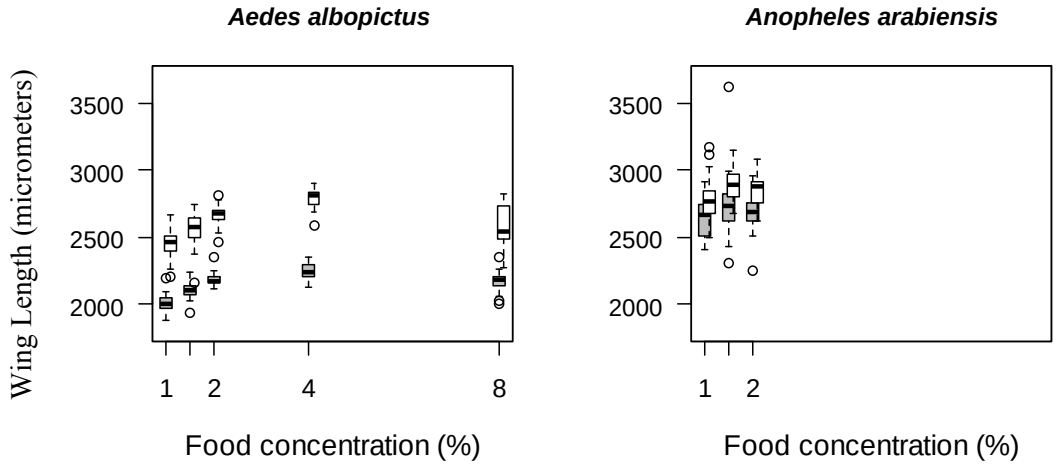

Figure 46. Box plots of wing length measurement for *Aedes albopictus* and *Anopheles arabiensis*. Males and females wing length measurements are represented respectively by the grey and white boxes. Circles represent outliers (An outlying observation, or outlier, is one that appears to deviate markedly from other members of the sample in which it occurs).

The 2% diet resulted in good survival, short development time and enhanced the size difference between sexes and is therefore recommended for larval rearing of *Ae. albopictus.*

However for *An. arabiensis*, the growth parameters were improved when 1% of diet was used. For both species, the effect of all these improvements and specific modifications on the sexual competitiveness of the males will need to be assessed in semi-field settings and in field trials.

3.4.6. Effect of Laboratory-Rearing Conditions on Male Anopheles arabiensis *Sexual Maturation*

Male mosquitoes are not sexually mature immediately after emergence and they emerge with terminalia rotated towards their dorsal side. A 180° rotation of this part is needed for them to be able to mate. Depending on the mosquito species, the rotation takes from 6 to 40 hrs. It was generally assumed that *An. arabiensis* males would not be able to mate during the first 24 h after emergence.

Obtained data that were difficult to explain from experiments involving mating of virgin females with radio-sterilised males suggested that males originating from the reared strain *An. arabiensis* Dongola could be sexually mature earlier than 16 h after emergence. Therefore, an experiment was conducted where male and female adults of the same age were left together in cages for 11, 12.5, 14, 15.5, 17 or 19.5 h after emergence. Observations of male terminalia rotation (**Figure 47**) indicated that most of the males were sexually mature and already able to mate 11 h after emergence (**Figure 48**). Spermatheca dissections confirmed that more than 78% of the females present in the cage were inseminated after this amount of time.

In 2007, this phenomenon was not reported in this strain, as females separated from males less than 18 h after emergence were still virgin. Completion of each *An. arabiensis* generation takes approximately 20 days (from eggs hatching to fathering adults), which will amount to about 18 generations/yr. This study of male sexual maturation was

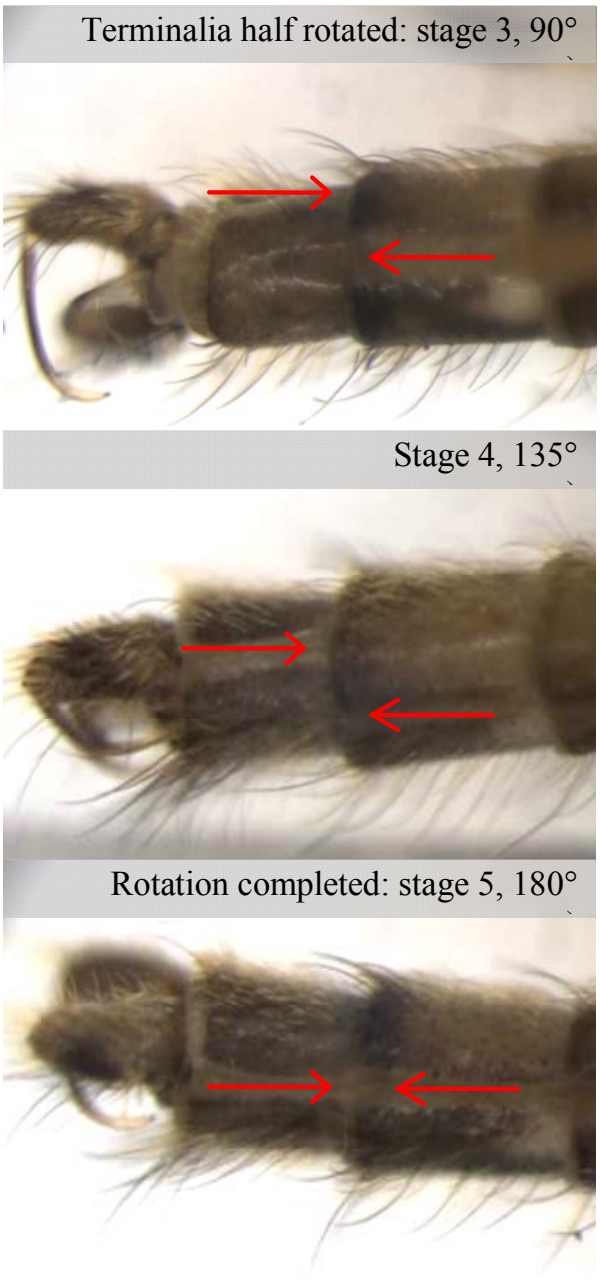

Figure 47. Three different stages of male terminalia rotation (*Anopheles. arabiensis*); males are considered capable of mating when rotation attains at least 135°

conducted with generation F_{124} and therefore, sufficient time has elapsed since the first generations of reared *An. arabiensis* Dongola so that selective pressure caused by laboratory conditions could have favoured earlier sexual maturation of the male mosquitoes.

Such a selection has previously been reported for fruit flies but we found no record concerning mosquito species. Important consequences result from this: particular care must be taken with laboratory experiments involving sterility and purifying crosses between Dongola virgin females and GSS males; however this early maturation of the males would be an advantage to the release of reared sterile males in the field.

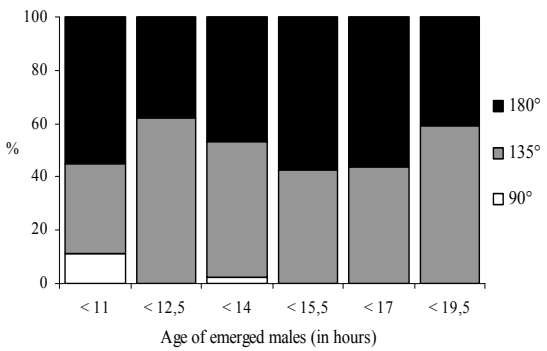

Figure 48. Percentage in each of the three last stages of terminalia rotation, for the different groups of age in colony strain *Anopheles arabiensis* Dongola males.

3.4.7. Male Anopheles arabiensis *Flight Performance Assessed in Flight Tubes*

A device (flight tube) was designed to assess differences in flight performances of *An. arabiensis* individuals subjected to different treatments (e.g. irradiation) or fed with different diets. The flight tube consisted of 8 vertical levels of transparent plastic cylinders (12 cm diameter x 20 cm high), linked together by an upside-down funnel, which allowed mosquitoes to fly to the upper compartment but prevented them from flying to a lower compartment (**Figure 49**). Male mosquitoes were placed in a cup as pupae in the lower compartment; no sugar source was offered. The day following emergence, the number of adults in each level was recorded hourly, and a final record was taken the next day.

Figure 49. Flight tubes.

Control tests showed that three groups of males originating from the same batch behaved in a similar way. Males irradiated as pupae at 70 or 120 Gy were then compared with untreated males. All the replicates showed the same pattern with control males reaching a higher mean height in the flight tube as compared to irradiated males (**Figure 50**). An increased number of males seemed to enhance these differences between the groups. Further experiments will be conducted in order to standardize the use of the flight tube for different conditions (number of males, presence of sugar) so

that similar analyses could be carried out in different laboratories as well as in the field. It still needs to be assessed whether a better flight performance can be correlated with a higher competitiveness. If a relationship is found between flight and competitiveness, the flight tube would be a good addition to field cages for testing competitiveness.

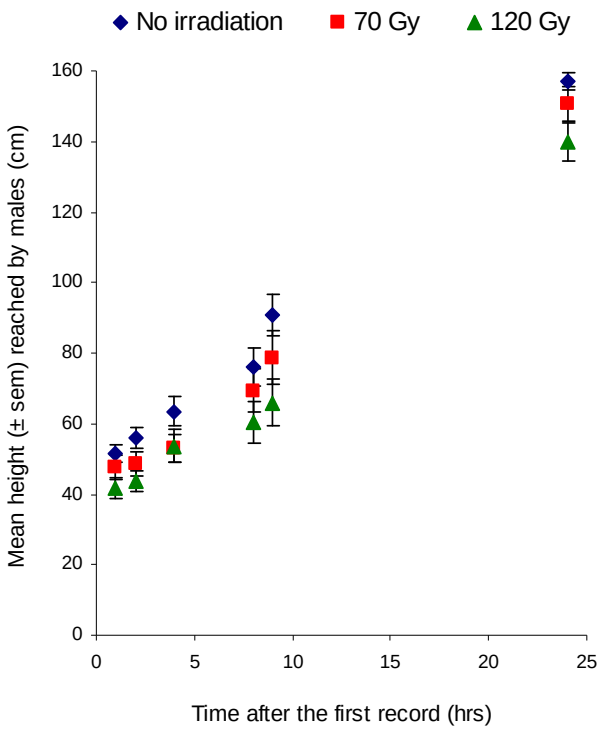

Figure 50. Mean height (± sem) reached by males over of time in flight tube.

3.4.8. Radioprotection

The success of programmes that incorporate an SIT component depends mainly of the competitiveness of the released male insects. However the irradiation process can induce a loss of competitiveness due to somatic damage. Some chemicals have shown radioprotective capacities, extending the lifespan of irradiated mammals or reducing damage induced by radiation. Some of these chemicals were tested for improvement of the competitiveness of irradiated mosquitoes. Preliminary experiments showed a good radioprotective potential of three chemicals used as additive to the larval diet: Nordihydroguaiaretic acid (NDGA), chitosan and bee venom. Results suggested that, when fed at the larval stage, irradiated males in competition with non-irradiated males competed better for females than irradiated males fed on a normal diet. However virginity of the females used in this experiment was not ensured due to the problem of early sexual maturation of the males (see 3.1.4.6). Further tests are planned to confirm these results.

3.4.9. Larval Tray Colour and Colour Marking

A selective marker for released sterile male mosquitoes would be needed so that dispersal, dispersion and density of the population could be assessed after release. We investigated the possibility of colour-marking adult mosquitoes using differently coloured larval rearing trays (**Figure 51A**). Larvae reared in black or white containers showed a different pigmentation corresponding to their environment background colour. This phenomenon, called homochromy, was observed for the larvae as well as for the emerging adults.

The different background colours did not influence significantly the growth parameters. A picture analysis study (**Figure 51B**) was done on *An. arabiensis* to assess whether the pigmentation is uniform enough to enable us to use this induced pigmentation as a marker for released mosquitoes. Analysis of Red Green Blue pixels showed a good separation between mosquitoes reared in black and white petri dishes (**Figure 51C**). Wild mosquitoes from La Réunion were analyzed as well and they mostly overlapped with the group of mosquitoes reared on black underground; breeding sites of La Réunion are mainly found in dark lava rocks. This result suggests that the white coloration could be used to mark sterile male mosquitoes for release in La Réunion. However so far, the image analysis used indicates that only a rough estimation was possible as some mosquitoes from La Réunion overlapped with mosquitoes reared on white background. Further images analyses will be needed to confirm that this method could actually be used for mark-release purposes. In addition, comparisons between wild mosquito populations from different breeding sites of La Réunion and Sudan are needed to assess the level of homogeneity in the wild population coloration.

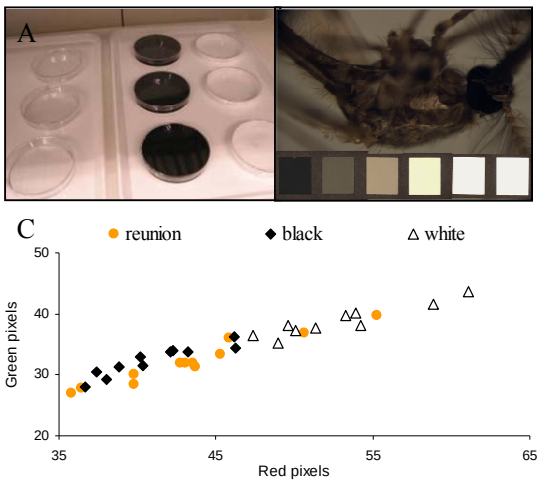

Figure 51. (A) The Petri dishes with the different coloured background, (B) male mosquito with colour calibration cards and (C) pixel analysis of male mosquitoes reared on a black or white background and wild mosquitoes from La Reunion.

4. APPENDICES

4.1. Personnel Changes

The Insect Pest Control Laboratory was joined for six weeks by two American scientists (Mr Guy Hallman and Mr Scott Myers) who carried out studies on the effects of cold treatments of fruits on fruit flies. The tsetse group received the consultants Mr Gratian Mutika, Zimbabwe (for 7 months), to assist with mating behaviour studies of *Glossina palpalis gambiensis* and Mr Henri Kariithi, Kenya (for 6 months) to assist with the work on the *Glossina pallidipes* virus. Mr Ulyses Sto Thomas was recruited in August on a temporary basis to provide technical assistance with the rearing of tsetse. Mr Alan Robinson was again recruited for 6 months to help with the editing of various documents. Mr Pablo Liedo and Ms Dina Orosco left the Insect Pest Control Laboratory after having worked for a year on olive fly rearing and on the selection of beneficial traits for fruit fly SIT. Mr Mark Benedict left the mosquito group in mid 2009 and returned to the USA. Mr Rudy Boigner retired from the group in July 2010 after having served the unit for more than 25 years. He was a real asset to the group and we wish him all the best in his retirement.

4.2. Publications

In press

ABD-ALLA, A.M.M., D.G. BOUCIAS and M. BERGOIN Hytrosaviruses: Structure and Genomic Properties, pp. In Press. *In* Asgari, S. and K. Jonasson (eds.), Insect Virology. Horizon Scientific Press.

ABD-ALLA, A.M.M., H. KARIITHI, A.G. PARKER, A.S. ROBINSON, M. KIFLOM, M. BERGOIN and **M.J.B. VREYSEN**. Dynamics of the salivary gland hypertrophy virus in laboratory colonies of *Glossina pallidipes* (Diptera: Glossinidae). Virus Research.

FELDMANN, U., **A. PARKER**. The role of the sterile insect technique (SIT) in tsetse control - using a pest to attack itself. Public Health Journal.

MEHTA, K., **A. PARKER.** Characterization and dosimetry of a practical x-ray alternative to self-shielded gamma irradiators. Radiation Physics and Chemistry.

SIMMONS, G.S., J. CARPENTER, M. SUCKLING, M. ADDISON, A. DYCK and **M.J.B. VREYSEN**. Improved quality management to enhance the efficacy of the sterile insect technique for lepidopteran pests. Journal of Applied Entomology.

TARET, G., M. SEVILLA, **V. WORNOAYPORN, A. ISLAM, S. AHMAD**, C. CACERES, **A.S. ROBINSON** and **M.J.B. VREYSEN**. Mating compatibility among populations of codling moth *Cydia pomonella* Linnaeus (Lepidoptera: Tortricidae) from different geographic origins. Journal of Applied Entomology.

VREYSEN, M.J.B., J.E. CARPENTER and F. MAREC. Improvement of codling moth *Cydia pomonella* (Lepidoptera: Tortricidae) SIT to facilitate expansion of field application. Journal of Applied Entomology.

BOUYER, J., S. RAVEL, L. GUERRINI, JP. DUJARDIN, I. SIDIBE, **M.J.B. VREYSEN**, P. SOLANO, and T. DE MEEUS Population structure of *Glossina palpalis gambiensis* (Diptera : Glossinidae) between river basins in Burkina Faso: consequences for area-wide integrated pest management. Infection, Genetics and Evolution.

2009

ABD-ALLA, A., F. COUSSERANS, **A. PARKER**, M. BERGOIN, J. CHIRAZ and **A. ROBINSON** (2009). Quantitative PCR analysis of the salivary gland hypertrophy virus (GpSGHV) in a laboratory colony of *Glossina pallidipes*. Virus Research. 139:48-53.

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BENEDICT, M.Q., B.G.J. KNOLS, H.C. BOSSIN, P.I. HOWELL, E. MIALHE, C. CACERES and **A.S. ROBINSON** (2009). Colonisation and mass rearing: learning from others. Malaria Journal. 8(Suppl 2):S4.

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GARCIA-MARTINEZ, V., E. HERNANDEZ-ORTIZ, C.S. ZEPETA-CISNEROS, **A.S. ROBINSON**, A. ZACHAROPOULOU and **G. FRANZ** (2009). Mitotic and polytene chromosome analysis in the Mexican fruit fly, *Anastrepha ludens* (Loew) (Diptera: Tephritidae). Genome. 52(1):20-30.

GARCIA-MARUNIAK, A., **A.M.M. ABD-ALLA**, T.Z. SALEM, **A.G. PARKER**, M.M. VAN OERS, J.E. MARUNIAK, W. KIM, J.P. BURAND, F. COUSSERANS, A.S. ROBINSON, J.M. VLAK, M. BERGOIN and D.G. BOUCIAS (2009). Two viruses that cause salivary gland hypertrophy in *Glossina pallidipes* and *Musca domestica* are related and form a distinct phylogenetic clade. Journal of General Virology. 90:334-346.

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PAPATHANOS, P.A., H.C. BOSSIN, **M.Q. BENEDICT**, F. CATTERUCCIA, C.A. MALCOLM, L. ALPHEY and A. CRISANTI (2009). Sex separation strategies: past experience and new approaches. Malaria Journal. 8(Suppl 2):S5.

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4.3. Travels

4.3.1. Staff

Name	Destination	Period	Purpose of Travel
Abd Alla, Adly	Bobo Dioulasso, Burkina Faso	9-20 February 2009	To attend a workshop on DNA isolation and detection of tsetse pathogens and symbionts using polymerase chain reaction (PCR) and to organise the 2nd RCM on insect symbionts and pathogens.
	Montpellier, France	9-13 March 2009	To collaborate with Prof. Max Bergoin to clone the complete genome sequence of SGHV into bacterial artificial chromosome (BAC) and to purify the SGHV on Nycodenz gradient.
Balestrino, Fabrizio	Turin, Italy	9-13 March 2009	To attend the 5th European Mosquito Control Association Meeting (EMCA) and present a poster on "Novel designs for mass rearing equipment being developed and constructed at IAEA Seibersdorf Laboratories".
	Bologna, Italy	21-25 September 2009	To attend the 3rd Co-ordinated Research Programme Meeting on "Development of standardised mass rearing system for male Anopheles arabiensis mosquitoes" as IAEA Scientific Secretary.
Jessup, Andrew	Tokyo, Japan	26-30 January 2009	To participate in the Technical Panel on Phytosanitary Treatments under the International Plant Protection Convention.

Name	Destination	Period	Purpose of Travel
	Cordoba, Spain	1-4 June 2009	To participate in the Fourth Meeting of the IOBC Working Group on Integrated Plant Protection in Olive Crops and the First Co-ordination Meeting on Technical Cooperation Project RAS5051: Developing Integrated Control of the Olive Fruit fly.
	Pereybere, Mauritius	21-25 September 2009	To participate in the final RCM of CRP on Development of Mass Rearing for New World (*Anastrepha*) and Asian (*Bactrocera*) Fruit Fly Pests in Support of Sterile Insect Technique (SIT).
	Bet Dagan, Israel	14-19 November 2009	Review Meeting on Technical Cooperation Project RAS5051: Developing Integrated Control of the Olive Fruit fly Review progress of project.
	Amman, Jordan	19-23 November 2009	Review Meeting on Technical Cooperation Project RAS5051: Developing Integrated Control of the Olive Fruit fly Review progress of project.
Parker, Andrew	Ouagadougou, Burkina Faso	9-13 March 2009	Technical review mission (ETH5015) to assist the counterpart with rearing in module 1&2 at the Kaliti facility including assistance with resolving any problems with running or equipment of the modules
	Addis Abeba, Ethiopia	8-12 June 2009	Technical review mission (ETH5015) to assist the counterpart with rearing in module 1&2 at the Kaliti facility including assistance with resolving any problems with running or equipment of the modules.

Name	Destination	Period	Purpose of Travel
Vreysen, Marc	Hluhluwe, KwaZulu Natal (South Africa), Maputo (Mozambique)	23-27 March 2009	To participate in the first regional technical meeting of the joint Mozambique/South Africa tsetse control project (RAF5059) and to review activities in Mozambique.
	Christchurch, New Zealand	27 April-1 May 2009	To participate as the scientific secretary in the first RCM of the CRP on "Increasing the efficiency of Lepidoptera SIT by enhanced quality control".
	Dakar, Senegal	18-22 May 2009	To review the progress in project SEN5031.
	Antwerp, Belgium	15-16 June 2009	To participate in the Project Management Committee and Scientific Meetings of the EC-INCO and Bill Gates Foundation.
	Dakar, Senegal	5-9 October 2009	To participate in the workshop on the development of an operational research plan for the desert locust in Western Africa.
	Thiès, Senegal	12-16 October 2009	To review TC project SEN5031 and to participate in the national workshop on suppression techniques
	Indianapolis, Indiana, USA	13-16 December 2009	To participate in the 57th Annual Meeting of the Entomological Society of America and to present a lecture at the SIT Symposium organised by the Insect Pest Control Sub-programme in collaboration with colleagues of USDA-APHIS.

4.3.2. Consultants

Name	Destination	Period	Purpose of Travel
Gilles, Jeremie	Turin, Italy	9-13 March 2009	To attend the annual meeting of the European Mosquito Control Association and present a poster on "Experimental investigation of negative and positive density dependence effects in *Anopheles arabiensis* larval competition"
	La Réunion, France	23-27 March 2009	To attend the Kick-Off meeting of the SIT mosquito project
	Paris, France	28-29 September 2009	To attend a meeting to elaborate a response to an FP7 call for Africa Health 2010.2.3.2.4. A project is being poroposed by IRD entitled: "New operational tools and methods to control malaria vectors in Africa – NOMALVEC"
	Antalya, Turkey	11-16 Oct 2009	To attend the 5th international Society of Vector Ecology Meeting and present a poster Presentation "Diet formulation for *Anopheles arabiensis* and *Aedes albopictus* aquatic stages"
	La Réunion, France	7-24 December 2009	To attend a Meeting with the Indian Ocean Commission and the countries of the area (Mauritius, Seychelles, Madagascar, and Comoros) to present the feasibility study for a SIT programme in LA Reunion and the implication on the regional level.
Kabore, Idrissa	Dakar, Senegal	5-9 October 2009	To assist in the installation of the rearing equipment and making the dispersal centre operational (Project SEN5031)
Oliva, Clelia	La Réunion, France	11-30 December 2009	To attend a meeting with members of the SIT Project

4.4. External Collaborations and Partnerships

Institution	Topic
Center for Medical, Agricultural, and Veterinary Entomology, U.S. Department of Agriculture Agricultural Research Service, USA	Pheromone analysis and transgenesis
Department of Developmental Biology, Johann-Friedrich Blumenbach Institute of Zoology and Anthropology Georg-August-University Goettingen Justus-von-Liebig-Weg 11, 37077 Goettingen	Transgenesis
Department of Entomology and Nematology, University of Florida, 970 Natural Area Drive, Gainesville, Florida 32611-0620, USA	Tsetse virus
Moro, Vancurova 12, 83101, Bratislava, Slovakia	Tsetse mass rearing
Institute of Zoology, Department of Entomology, Slovak Academy of Science, Dubravska cesta 9, 84506 Bratislava, Slovakia	Tsetse colony maintenance
CDFA, 3288 Meadowview Rd., Sacramento, CA 95832, USA	Olive fly rearing
Department of Biology, University of Crete, P.O. Box 2208, Vasilika Vouton, Heraklion, Crete, Greece	Olive fly rearing
SENASA, Av. La Molina 1915, La Molina, Peru	*Anastrepha* mating studies
Rad Source Technologies, Inc., 6825 Shiloh Rd East Ste B-2, Alpharetta, GA 30005, USA	X-ray machine
Iatros Ltd, Delta House, Gemini Crescent, Dundee Technology Park, Dundee DD2 1SW, Scotland	UV irradiation
Institute of Plant Protection, Agricultural Research Organization, P.O. Box 6, Beit-Dagan 50250, Israel	Olive fly radiation biology
Laboratoire de Pathologie Comparée, Université Montpellier II, France	Tsetse virus
Department of Forest & Soil Sciences, Forest Pathology & Forest Protection; BOKU, University of Natural Resources & Applied Life Sciences, 1190 Vienna, Austria	Juvenile hormone treatment
Department of Environmental and Natural Resources Management, University of Ioannina, 2 Seferi St., 30100 Agrinio, Greece	Wolbachia studies
Engineering Research Unit, USDA ARS Grain Marketing and Production Research Center, 1515 College Ave, Manhattan, KS 66502, USA	Tsetse pupal sexing
Department of Computing, School of Engineering and Physical Sciences, University of Surrey, Guildford, Surrey, GU2 7XH, UK	Tsetse pupal sexing

PBARC, USDA-ARS, P.O. Box 4459, Hilo, HI, 96720, USA	Fruit fly liquid diet
Programa Moscafruta, National Campaign Fruit Flies DGSV-SAGARPA, México, Av. Central Poniente No. 14, Tapachula, Chiapas, México CP 30700	*Anastrepha ludens* cytology
Epidemiology Department, Tropical Medicine Research Institute, P.O. Box 1304, Khartoum, Sudan	Mosquito field work

4.5. Trainees, Fellows and Scientific Visitors

Name	Country	Duration	Topic of Training
Fellows			
Sookar, Mr. P.	Mauritius	6 months	Fruit fly rearing and genetic sexing
Kinyosi, Mr. B.W.	Kenya	10 days	Dosimetry
Obore, Mr. P.O.	Kenya	10 days	Dosimetry
Dias, Ms. V.	Brazil	2.5 months	Fruit fly rearing
Kariithi, Mr. H.M.	Kenya	3.5 months	Tsetse virus
Khan, Mr. I.	Pakistan	3.2 months	Mosquito rearing
Ahmadi, Mr. M.	Iran	3 months	Fruit fly genetic sexing
Idris, Mr. E.	Syria	1 month	Mosquito rearing
Scientific Visitors			
Wamwiri, Ms. F.N.	Kenya	1 week	Tsetse rearing
Wiryosoetikno, Ms. W.	Indonesia	2 weeks	Mosquito rearing
Ukulli, Mr. J.	Tanzania	1 week	Tsetse rearing

4.6. Co-ordinated Research Projects (CRP)

Project Number	Co-ordinated Research Projects	Scientific Secretary
D4.10.20	Improving Sterile Male Performance in Fruit Fly SIT programmes (2004-2009)	Jorge Hendrichs
D4.10.21	Development of Mass Rearing for New World (*Anastrepha*) and Asian (*Bactrocera*) Fruit Flies (2004-2009)	Andrew Jessup
G3.40.01	Development of Standardised Mass Rearing Systems for Male Mosquitoes (2005-2011)	Fabrizio Balestrino
D4.20.12	Improving SIT for Tsetse Flies through Research on their Symbionts and Pathogens (2007-2012)	Adly Abd Alla
G3.40.02	Biology of Male Mosquitoes in Relation to Genetic Control Programmes (2008-2013)	Marc Vreysen
D4.20.13	Applying Population Genetics and GIS for Managing Livestock Insect Pests (2008-2013)	Udo Feldmann
D4.10.22	Increasing the Efficiency of Lepidoptera SIT Through Enhanced Quality Control (2009-2014)	Marc Vreysen
D6.20.08	Development of Generic Irradiation Doses for Quarantine Treatments (2009-2014, managed by Food and Environmental Protection Subprogramme)	Andrew Parker (co-secretary)
D4.20.14	Development and Evaluation of Improved Strains of Insect Pests for SIT (2009-2014)	Gerald Franz

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