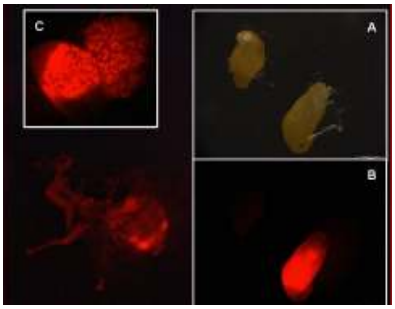
Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

Entomology Unit

Activities Report

2007







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The expansion of SIT programmes is resulting in an increased demand for traditional ^{60}Co radiation sources just as the major supplier of these sources is ceasing production. This has become an urgent and serious issue for many programmes. In the very early days of SIT, radiation for experimental purposes was carried out using X-rays as many laboratories did not have access to isotopic sources. Unfortunately, the penetration power of X-rays is much lower than that of isotopic sources and so it was never used in programmes. Recently new X-ray machines are being produced which are able to deliver X-rays with sufficient penetration for irradiation of large volumes of insect pupae or adults. The Unit acquired such a machine in late 2007 and testing was started. Initial testing has been hampered by frequent breakdowns of the machine and a much smaller potential irradiation volume than originally foreseen and only physical dosimetry has been partially carried out. The manufacturer will install a new X-ray tube in early 2008 so that testing can be continued.

One major advantage of the use of SIT is that it is species specific and this implies that the target population is the same species as the sterile flies being released. A major agricultural pest in South and Central America is the South American fruit fly Anastrepha fraterculus *and there is interest in developing SIT for this species. However, there are many reports that this nominal species is in fact a species complex. Work carried out in the Unit strongly supported these reports using two populations from Peru and Argentina. Major differences have been observed in sex pheromones, mating compatibility and chromosome karyotype of the two populations together with significantly reduced viability in certain crosses and sex ratio distortion. All this information strongly suggests that these two strains belong to different biological entities. This same situation might also apply to another economically important fruit fly* Bactrocera dorsalis *and similar work on this species complex is planned to be carried out in 2008.*

Genetic sexing strains have been shown to provide significant advantages to medfly SIT programmes and many years ago potential genetic sexing strains based on a white pupa mutation were isolated in two other fruit flies, Bactrocera dorsalis *and* Bactrocera cucurbitae*. A detailed genetic and cytogenetic analysis has now been completed for these two strains. For* B. dorsalis *a single autosome is involved in the translocation but there are multiple chromosome breaks in the male determining chromosome. The strain is relatively stable with an overall fertility of about 50%. For* B. cucurbitae *a very complex rearrangement could be observed with two autosomes involved and multiple breakpoints. The complexity of the rearrangement reduced the overall fertility of the strain to 22%, far too low to be of use in an SIT programme. For mosquito SIT, elimination of females is essential as the sterile females can still transmit the disease and a genetic sexing strain has been isolated in* Anopheles arabiensis*. It is based on male resistance to a particular dose of insecticide which is added to the larval rearing trays. The strain appears to be very stable and has already provided promising results in a field release in Sudan.*

Transgenic technology offers new opportunities to improve components of the SIT and the Unit continues to be involved in the development of new strains using this technology. In 2007 the work was expanded to include studies on mass rearing and field cage evaluation of several different transgenic strains. In general the results showed that the strain behaved reasonable well in both environments and the sexing strains tested were stable and produced only male progeny. This is encouraging but much more work needs to be done to confirm these preliminary results. An important step towards the eventual release of transgenic insects was the publication of a standard by the North American Plant Protection Organisation which provides a regulatory framework for the use of transgenic insects in plant protection programmes in the USA, Canada and Mexico.

Since the publication in 1992, of the Laboratory Training Manual on the Use of Nuclear Techniques in Insect Research and Control the global scientific environment has changed due to technological advances, such as molecular biology, computing etc. There have also been significant changes in the external political arena and from an environmental and political perspective, it is no longer acceptable to release radio-nuclides in to the field. Fortunately stable isotopes provide us with an alternative nuclear tool for studying the biology and ecology of insects, they are non-radioactive, do not decay, do not emit radiation and occur naturally in the environment. A draft update of the Laboratory Training Manual has been produced which provides a comprehensive introduction to the use of stable isotopes in an entomological context providing a step by step guide on how to use stable isotopes for a range of biological and ecological studies.

There have been many personnel changes during 2007. Mr. Mark Benedict returned to the Unit to lead the mosquito SIT project and Mr. Diego Segura was recruited to support the fruit fly work with the departure of Mr. Carlos Caceres to the Moscamed Programme in Guatemala. In March 2008 Mr. Andrew Jessup from Australia will take up the vacant position left by Mr. Caceres. To support the work on the olive fly, Mr. Ioannis Dimou spent two months in the Unit as a consultant. To support the activities related to the evaluation of transgenic strains, Mr. Neil Morrison from Oxitec spent 6 months in the Unit. In December 2007, Mr. Daniel Briceno joined the Unit on a one year sabbatical to study mating behaviour in tsetse flies. In February, Mr. Janis Thailayil enrolled for a PhD at Imperial College, UK and Ms. Michelle Helinski returned to the Netherlands to complete her PhD. At the end of 2007, Mr. Colin Malcolm and Ms. Taif Adams returned to Queen Mary College, London. In November 2007, Mr. Alan Robinson retired from the Agency after a 14 year period as Unit Head and Mr. Marc Vreysen will take his place in March 2008.

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1. PROGRAMMATIC AND UNIT OBJECTIVES

The vision and goal of the Insect Pest Control Section are to increase food security and to fight hunger through the development of the Sterile Insect Technique for area-wide integrated pest management programmes (AW-IPM) 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 insect pests of crops through the integration of 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 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 for the use of the Sterile Insect Technique for the control of malaria transmitting mosquitoes

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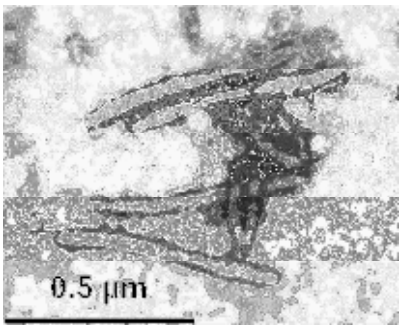
3. RESEARCH AND DEVELOPMENT ACTIVITIES

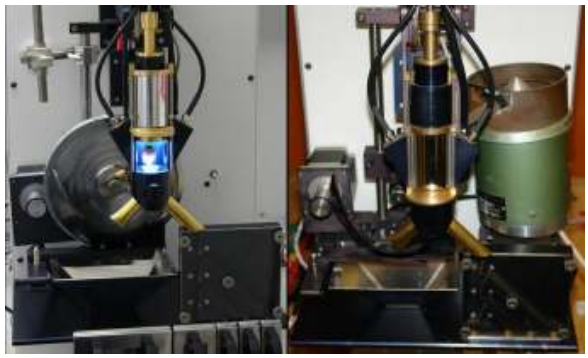
3.1. Tsetse Rearing and Virus Analysis

The Unit maintains several colonies of economically important tsetse species, in some cases these colonies are not available elsewhere and many institutions world-wide are provided with tsetse material. The total number of flies maintained during 2007 was about 30 000 breeding females. In 2007 a concerted effort was made to fully evaluate TPU3 using G. pallidipes *on this automated system. The evaluation revealed several design flaws which could be easily corrected and at the end of the year a colony of 5000 breeding females was being maintained. Any design changes made have also been communicated to the manufacturer in order that they can be introduced onto the TPU3 system already installed in Kaliti, Ethiopia.*

The genomic sequence of the tsetse salivary gland hyperplasia virus from the Glossina pallidipes *Uganda colony has been completed and annotated. The virus is a double stranded circular DNA virus, 190kb long and contains 161 non-overlapping reading frames. It differs significantly from all other related groups of viruses and it represents a novel group of insect viruses. Sequence comparison with the virus from Ethiopian flies has shown very few differences. A wider analysis of the virus in flies from other regions in Africa is underway. Knowledge of the genome will enable critical genes to be targeted for knock-out using RNA interference methods with the aim to reduce viral replication in flies. In addition experiments have been restarted on the feeding of anti-viral agents to flies to reduce viral load. In many of these experiments quantitative PCR is a key component of the analysis.*



Efficient automated sorting of male and female pupae would provide tremendous advantages to tsetse SIT programmes. Although the use of infra-spectroscopy has proved to be encouraging, several problems remain to be solved involving both hardware and software. In 2007 alternative data analysis software was used involving neural network principles and modifications have been made to the pupal feeder system on the machine. In 2008 these modifications will be tested and new software will need to be developed with the support of the manufacturer of the equipment.



3.1.1. Colony Status [1]

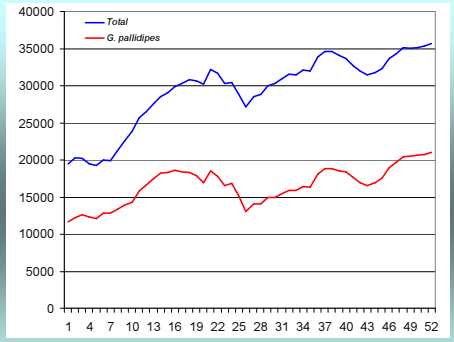


Figure 1. Total colony size and *G. pallidipes*.

The tsetse colonies have recovered well from the large fall late in 2006 resulting from equipment failure. All colonies showed good growth in the first half of the year **(Figure 1)** and, from midyear, the colony sizes have been limited to their target sizes of 5000 for *Glossina palpalis*, 4000 for *G. brevipalpis* and 15 000 for *G. pallidipes*. The *G. morsitans centralis* colony has meanwhile been reduced to 4000 to reduce the workload as the demand for this species is currently low.

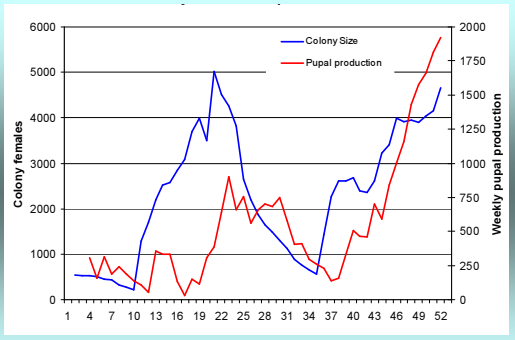


Figure 2. *G. pallidipes* colony on TPU3.

The good performance of the *G. pallidipes* colony has resulted in considerable excess material, which has been used to fill the TPU3 system. Input to the TPU3 system has risen from one frame (9 cages) per week to three or four by the end of the year. Performance on the TPU3 has been variable, with problems with the humidification system on several occasions leading to raised mortality. However, the performance has now steadily increased from about week 34 with a rapid improvement in pupal production **(Figure 2)**.

3.1.2. Pupal Sexing [2]

Tests to validate the pupal sex sorting using the SKNIR Spectrometer were continued. A new protocol was developed to accommodate the variability in sort value results previously observed (**2006 Activities Report**). Each day a sample of 100 pupae is first sorted to calculate the mid-point of the distribution and this is then used to adjust the sorting limits. The whole batch of pupae is then sorted using this value. From first principles the mid-point should be 1.5, half way between female (1.0) and male (2.0), but the actual values varied greatly from day to day **(Figure 3)**. Part of this variation comes from the age of the pupae with the mean sort value dropping as the pupae get closer to emergence, but within one age the variation is still substantial. The source of this variation is still not known.

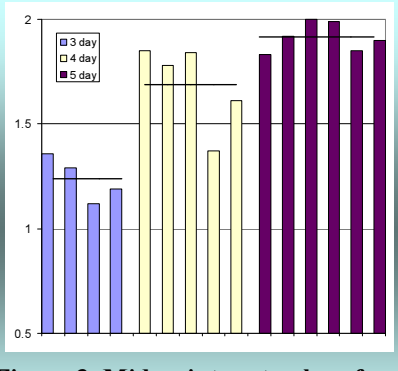


Figure 3. Mid-point sort values for 100 pupae. Means for each age shown by horizontal lines.

Preliminary results indicated that some variability in the sexing efficiency still exists after adjusting the mid point as above. Tests which have shown good pupae distribution, i.e. about 50% of the sexed batch being

[1] E2.04, activity 1
[2] E2.04, activity 7

sorted in bins 3 and 4, have unfortunately resulted in an unsatisfactory sex ratio of the emerged flies in bin 4. Compared to the desired minimum of 75% of male flies in bin 4, the proportions observed varied between 54% and 71%, mixed with 0.05 to 11% of female flies. Following a series of tests to improve the pupal sex sorting it was observed that a slight correction of the mid-point of the distribution of pupae batches leads to improved scanning results. In reducing the mid-point value by 0.05, the pupal sorting resulted in an increase in male flies (74.75 to 91.55%) and a decrease in females (1.06 to 3.96%) in bin 4.

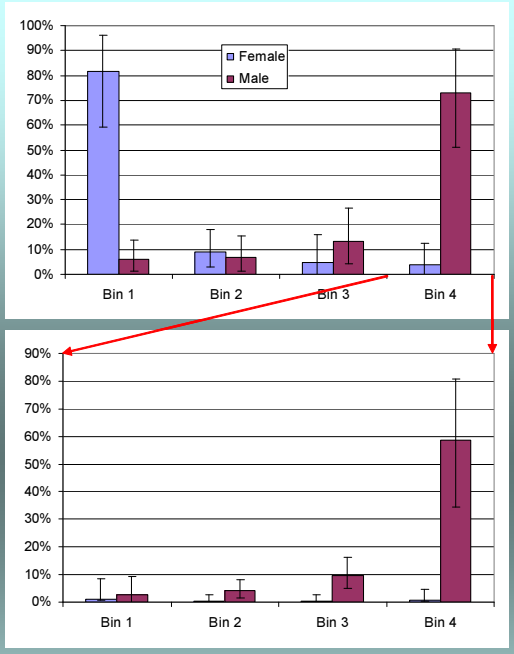


Figure 4. Pupal sexing results after adjusting the midpoint. The top graph shows the proportion of emerging females and males sorted to each of the four bins with 95% confidence intervals. The lower graph shows the distribution of emerging females and males when the pupae from bin 4 are sorted a second time using the same bin limits.

Following a suggestion from a visiting consultant, we also tried taking the pupae sorted to bin 4 and resorting them **(Figure 4)**. Some males are lost as they end in bin 1 or 2, but the proportion of females in bins 3 and 4 is reduced resulting in better sexing efficiency. We will continue to test this double sorting combined with reducing the mid-point value by 0.05. A significant constraint on this work has been the inefficient performance of the system to feed individual pupae into the spectrometer. The system is based on a vacuum pick-up wheel which is rotated by a stepping motor, the pupae are scraped off the vacuum pick-up and drop into the scanning bucket. This system was designed for picking up wheat grains, the original function of the SKNIR, but the vacuum pick-up holes easily become blocked by dust and dirt from the tsetse pupae. When this happens the machine wastes a lot of time waiting for the next pupa to be dropped into the measuring bucket. We are replacing this system with a vibration drive to feed pupae to a chute from where they drop to the reading bucket; as they drop they cut a light beam to stop the vibration drive until the spectrum has been read. This has the advantage that the vibration drive will only stop once a pupa has dropped and under normal circumstances another pupa will be ready to drop immediately saving considerable time. We presume that using the new protocol, based on calculation of the mid-point, it will be possible to sex and sort at least 75% of male pupae in bin 4 with minimum females, which will be easily separated and irradiated for field release.

3.1.3. Preliminary Evaluation of the X-ray Irradiator [3]

As reported in the previous **Activities Report (2006)** the Entomology Unit purchased an RS2400 X-ray irradiator from RadSource Technologies of Alpharetta, Georgia, USA. The unit was delivered and installed in September. Our interest in an X-ray system was to replace the use of cobalt-60 based irradiators for insect sterilization, as the purchase, transport and securing of

[3] Unplanned activity

isotopic irradiators is becoming more and more difficult, and the main supplier of such small irradiators withdrew their product in 2006.

The RS2400 as delivered to us has a maximum energy of 150 keVp with a variable current of 0-35 mA for a maximum total power just over 5 kW. Our unit was supplied with its own external water cooling unit to remove the waste heat from the X-ray tube **(Figure 5)**. It was supplied with five cardboard irradiation canisters with plastic end caps, with internal dimensions of 178 mm diameter and 167 mm long to give an effective combined volume of a little over 20 litres. Each irradiation canister is suspended in a cradle so that it may revolve around the horizontal X-ray tube with spacing from the surface of the tube of about 10 mm, providing irradiation from all sides of the canister **(Figure 6).**

Figure 5. Water cooling system for the x-ray machine.

Figure 6. Irradiation canister in its cradle.

The two main issues arising with the RS2400 relate to reliability and dose rate and distribution. The X-ray tube supplied with the machine has failed repeatedly, when the high voltage has arced across the face of the insulator in the base of the tube. This causes the voltage from the high voltage power supply to collapse, and the voltage can not recover due to the conducting path formed by the arc. The only solution to this is to replace the sealing washer and silicone grease and remake the connection. This takes approximately 30 minutes. However each time an arc occurs it causes further damage to the insulator, making it more likely that a new arc will form, and eventually it became impossible to use the machine. This problem should be completely resolved when a new X-ray tube is fitted.

Figure 7. Radiation canister with rice and a dosimeter.

The second issue relates to dose distribution and dose rate. In the original specification a dose rate of 45 Gy min^{-1} was given, but the dose rate to the centre of a 180 mm diameter PMMA canister (wall thickness 3 mm), 200 mm long spaced about 10 mm from the X-ray tube with instant rice as a dummy **(Figure 7)** (0.4 g cm^{-3}) was only about 16 Gy min^{-1}. Further, and more importantly, the dose distribution within this volume is very poor, with the ratio of maximum to minimum dose (DUR, dose uniformity ratio) about six. In a normal production environment a DUR of 1.4 would be acceptable.

The dose rate and DUR will vary with the diameter of the canister, density of the material being irradiated and distance from the tube. The density of the material cannot be changed, but the other two factors can. Dose rate and DUR will both improve (dose rate increase, DUR decrease) in a smaller diameter canister, whilst dose rate will decrease when the canister is further from the X-ray tube but the DUR will improve. We therefore decided to try a 120 mm diameter canister placed as far from the X-ray tube as the machine permits, giving a spacing of about

70 mm. In this configuration the central dose rate was about 20 Gy min^{-1} and the dose uniformity is greatly improved, with a DUR of about 1.6 **(Figure 8)**. The central region has a very good dose distribution (±5% in the central region 7 cm diameter by 10 cm long) and the dose rises only slowly towards the outside of the canister (+15%), but the fall in dose towards the ends is much greater (-25-30%). To obtain the desirable DUR of 1.4 or better would require blocking off about 2 cm from each end of the canister to avoid the lowest dose areas **(Figure 9)** leaving a volume of a little more than 1.5 litres. The much smaller canister size (down from the original volume of four litres) is a significant issue as it will greatly increase the handling because it will require many small batches of pupae.

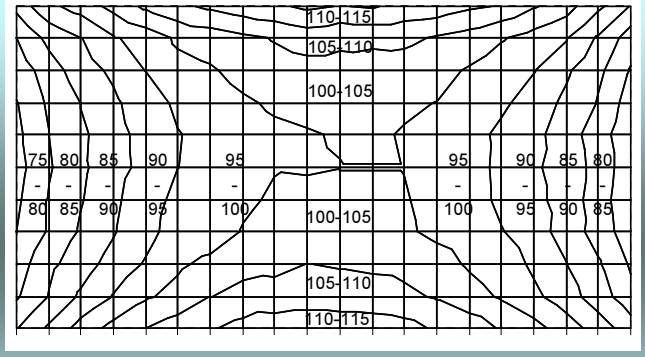


Figure 8. Dose distribution as a percentage of centre dose inside a 120 mm diameter canister, 200 mm long, aligned approximately 70 mm from the X-ray tube with instant rice (0.4 g cm^{-3}) as a dummy material. 1 cm grid.

As the dose only rises slowly in the centre line the working volume may be increased by increasing the diameter whilst reducing the length still further to obtain the desired DUR. We will test a 150 mm diameter canister once the X-ray tube is replaced, with thicker end walls to increase scatter. A volume of two litres or more may be possible, and with the increased spacing from the X-ray tube it should be possible to accommodate six or eight canisters per irradiation.

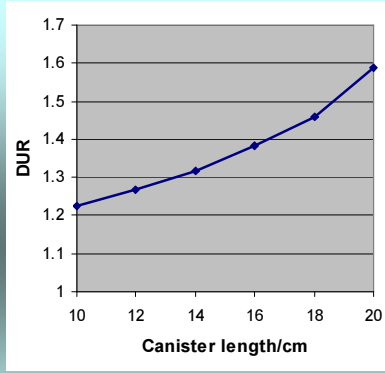


Figure 9. Dose uniformity ratio (DUR) as a function of canister length in the 120 mm diameter canister.

3.1.4. Salivary Gland Hypertrophy Virus [4]

As reported earlier some tsetse species carry a virus **(Figure 10)** that, in a certain proportion of individuals leads to salivary gland hypertrophy (SGH) and these individuals also show reproductive abnormalities. This SGH is present in natural populations at a low level (0.5-5%), based on salivary gland dissection and in a colony of *G. pallidipes* maintained in the Unit, the frequency of SGH ranges from 4-10%. However, PCR analysis has confirmed that virus prevalence is almost 100%. 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

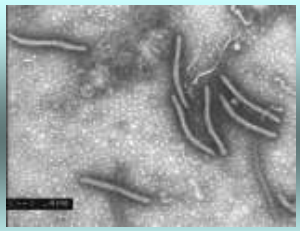


Figure 10. SGHV particles.

[4] E2.04, activity 12

about the virus with the goal to develop a management strategy for the virus. The most effective way to begin this study is to understand more about the virus in terms of its taxonomy and this can be done by obtaining nucleotide sequence information. Beside the sequence analysis the work aimed to gather information about the biology of the virus and its relation to the hypertrophy symptoms and sterility. Work on the effect of some antiviral drugs to suppress viral replication was also initiated.

3.1.4.1. Production of the SGHV by male screening

As reported in the **2005 Annual Report** virus production was attempted using artificial infection of 3rd instar larvae, but high mortality was observed and only 25% infected flies with enlarged salivary gland of the initially injected larvae were obtained. Moreover the long period of pupation (25 days at 25°C) makes production of the virus a very time consuming process. Due to the sex ratio used in tsetse colony maintenance, i.e. 1 male:4 females, a large number of males are not used in the rearing process and are normally discarded. Based on the observation that males with enlarged salivary glands can be distinguished by eye **(Figure 11)**, staff from the tsetse group were trained to screen males to select those with enlarged salivary glands all of which will be dissected later to collect hypertrophied glands for virus purification. The optimum age to screen males was identified and the best results could be achieved using 10-15 day old males and two day post feeding. Using this method a large number of hypertrophied salivary glands were accumulated for virus purification. The lack of a cell culture system is a major problem for virus production.

Figure 11. Male tsetse, A: normal SG, B: male with SGH.

3.1.4.2. Improvement of the virus purification procedure

We observed some difficulties with virus purification using linear sucrose gradient methods as shown by damage to the viral envelope in electron microscopy observations. Moreover the virus did not separate well to give a clear band and remained distributed over the gradient. To avoid these disadvantages we used the Nycodenz linear gradient of 10-60% to purify the virus particles. A clear white band was obtained as presented in **Figure 12**. The high quality of the extracted DNA was confirmed using spectrophotometry.

Figure12. Nycodenz gradient.

3.1.4.3. Confirmation of SGHV genome circularity by restriction endonucleases digestion

Despite the progress in sequencing the genome of SGHV, the nature of the virus genome as to whether it is circular or linear remained uncertain. To detemine the nature of the genome, restriction endonucleses enzyme digestion was used in combination with the genetic information available by the sequence data (**Figure 13**). The restriction endonucleases were selected based on the sequence analysis software which produce a reasonable number of DNA fragment. After digestion the DNA was checked on 0.8% gel agarose electrophoresis and the band, which

represents the junction between the start and the end of the sequence, was clearly observed. The presence of this band in the DNA digested with Dra II **(Figure 13, in red)** clearly confirm that the DNA is circular and this conclusion is in agreement with the sequence data.

3.1.4.4. Final genome sequence of SGHV

The genome sequence of SGHV originating from the Uganda *G. pallidipes* popualtion was completed. The GpSGHV genome is a double-stranded circular DNA of 190 032 bp containing 160 non-overlapping open reading frames (ORFs) distributed equally on both strands with a gene density of one gene per 1.2 kb. It has a high A+T content of 72%. About 3% of the SGHV genome is composed of 15 repeat sequences, consisting essentially of direct repeats, distributed throughout the genome. Predicted ORFs are evenly distributed on both strands (51% forward, 49%reverse), most commonly as clusters, and represent 86% of the genome. We arbitrarily assigned position 1 of the viral sequence to the A of the ATG translational initiation codon of the ORF encoding a protein similar to p74 occlusion-derived virion (*odv*) envelope protein of baculoviruses.

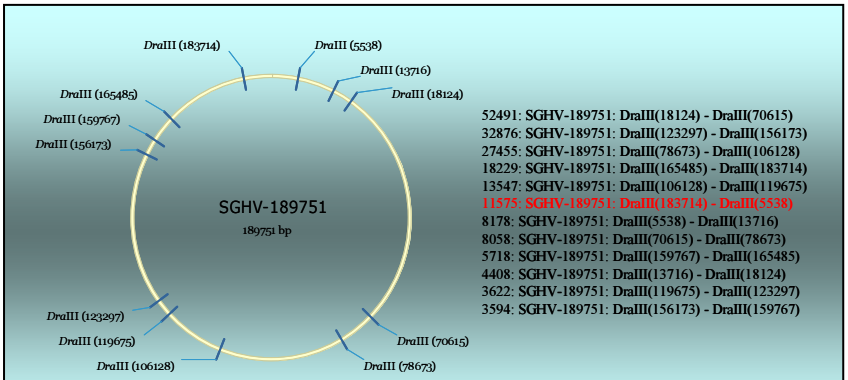


Figure 13 Restriction enzyme site of *Dra* III on SGHV genome

3.1.4.5. Genome sequence of SGHV from Ethiopia

Due to the possible importance of the virus infection for rearing *G. pallidipes* at the Kaliti facility in Ethiopia, and also to differences in the impact of the virus in the Ugandan flies as opposed to the Ethiopian flies, work has started on the partial sequencing of the virus strain from Ethiopia. The infected colony from Uganda has been in colonization for 20 years whereas the colony that was established from Ethiopia in Seibersdorf collapsed after two years. We have obtained sequence data that covers 95% of the genome, the genome length was estimated to be 189 769 nucleotides and shares 98.7% nucleotide identity with the Uganda isolate. This analysis will enable the identification of homologous genes against which to develop antibodies or target for RNA interference. Further it is important to determine the relationship between both isolates from an evolutionary standpoint and to determine if they are closely related strains of the same virus or represent two different viruses. The limited sequence differences so far found would indicate that the viruses are very closely related.

3.1.4.6. Impact of antiviral drugs on mortality and productivity

As reported in the **Activities Report 2006** we analysed the impact of two antiviral drugs on the virus replication in flies by the quantitative PCR. It was shown that there was a slight decrease in the virus copy number following antiviral drug treatment but the conclusions were not sufficiently clear. We have repeated the experiment with different concentration of the antiviral drugs firstly focusing on the impact of the antiviral treatment on the fly mortality and secondly on pupal production. Significant reductions in fly mortality were recorded for some of the treatments

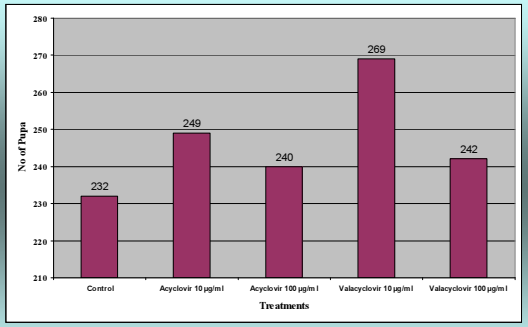


Figure 14. Impact of the antiviral treatmenton tsetse flies productivity.

accompanied by increases in pupal production (**Figure 14**). Further work will be undertaken to estimate the viral load in the flies following the different treatments. This will be done using quantitative PCR.

3.1.4.7. Virus prevalence in different field populations

All virus prevalence studies in the tsetse wild populations were carried out in the past based on the observation of the enlarged salivary gland. Recently we developed a PCR diagnosis method for the virus and the results showed that even though the prevalence of enlarged salivary gland was between 5-10% in the *G. pallidipes* colony, the virus prevalence based on the PCR was between 90-100%. These results indicate asymptomatic infection with the virus. There are no previous data available about the virus prevalence in the natural population of tsetse using the PCR diagnosis, as most of the previous studies were carried out based on the SGH observation. The obtained results indicate that the virus prevalence in the natural population of tsetse is 20-50% dependent on the location and the species (**Figure 15)**.

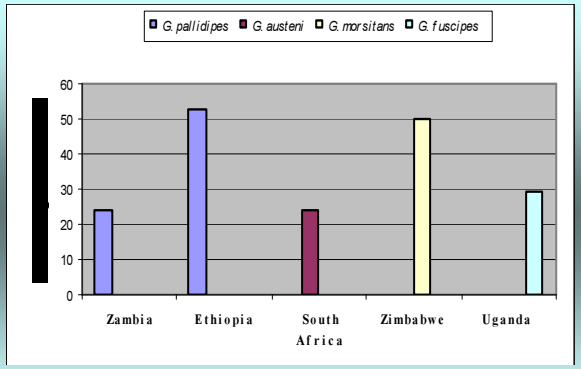


Figure 15. Virus prevalence in tsetse natural population in African countries.

3.1.5. Rearing *Glossina pallidipes* on TPU3.2 [5]

The Tsetse Production Unit version 3.2 (TPU3.2) is being evaluated in a routine tsetse mass-rearing manner, since 2007, in two laboratories, the Entomology Unit, Seibersdorf **(Figure 16)** and the Kaliti tsetse facility, in Ethiopia. The testing work in Seibersdorf, mainly aimed at improving the efficiency of this semi-automated tsetse holding and feeding system has previously lead to some technical adjustments, and to the introduction of a new design of cage to address problems with alignment and leveling

Figure 16. TPU3 with 5200 *G. Pallidipes.*

[5] E2.04, activity 13

of the cages on the membrane system. Tests pursued in the current year have resulted of important progresses in the use of the technology for the production of standardized, good quality reared flies in support of tsetse SIT application in Africa.

3.1.5.1. Improvements and modifications to TPU3.2 specifications

As results of the continuing implementation of the system, other minor modifications were made, such as changing the profile of the cage arm and replacing the aluminium saddle by moulded plastic supports for cage frame to improve stability and prevent rotation. A cable release for the feeding trolley locating mechanism was added in order to move it easier. Front and back clips **(Figure 17)** were also used to secure the feeding trays down flat on the heating plate to ensure homogeneity of the heating in all areas of the trays. Lighter collector slopes made of polycarbonate were introduced. Larger feeding trays adjusted to the maximum size available in the sterilizing oven and with strengthened edges were introduced. These trays will not bend following repeated washing and sterilizing. New larger feeding membranes (64.5 x 64.5 cm) were also produced to fit with the trays and to counteract the effect of the progressive shrinkage due to the heat during sterilization.

Figure 17. Feeding plate fixed with aluminium clips.

3.1.5.2. Fly feeding conditions and performances on TPU3.2

Following the minor changes to the system, 2480 *G. pallidipes* male flies aged 3 to 21 days, were loaded into four TPU3.2 frames and used to firstly confirm the expected positive effects. This has resulted in a good feeding response, an average daily mortality of less than 1.3% in all groups and a survival of 70% after 1 month observation. Tests with colony flies were started on 10th January 2007 with one frame of 540 *G. pallidipes* female and thereafter more frames were progressively loaded to confirm the improvements. A problem related to the climatic conditions was observed in the vicinity of the feeding station noticeable after about 1 hour of feeding. Humidity and temperature were subjected to variations affecting the flies of the 6th and following frames during the feeding process. The phenomenon was confirmed with the results of four frames of the same production unit, each loaded with 540 *G. pallidipes* female collected from the same emergence group. These frames fed respectively at the 5th, 6th, 7th and 8th position have shown different fly survival rates of 53.7%; 38.7%; 4.4% and 0.0% within the same observation period.

The problem clearly identified was a result of insufficient ventilation due to the size of the handling room and also due to the type of temperature regulator of the heating plate which resulted in more than 2ºC variation on top of the feeding membrane. The temperature regulator was thus modified by the supplier to have less than 1ºC variation on the feeding membrane. A mean temperature variation of 0.2ºC ± 0.1ºC was registered on top of the new feeding trays, in

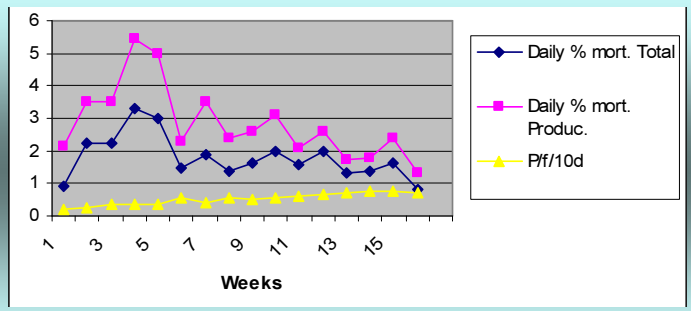


Figure 18. *G. pallidipes* performance on TPU3.

comparison to a value of 1.7 ± 0.78ºC previously found on the trays placed on the same heating plate before the modification. In addition to this improvement, the membranes were slightly humidified during fly feeding.

The situation was improved and flies fed better, survived better and performed better. The daily mortality was about 1.27 and the number of pupae/female/10 days was 0.73 **(Figure 18).** In December 2007, a total of about 5300 *G. pallidipes* were kept on the TPU3.2. The size of the colony was being progressively increased to confirm improvements in tsetse routine mass rearing on the semi automated system.

3.1.6. Dosimetry Inter-comparison Service [6]

In the application of the sterile insect technique, the performance of the flies in the field is critical. The performance of the flies can be reduced by many factors, such as poor rearing conditions, rough handling, and over irradiation. The dose required to produce a given level of sterility can be calculated, as can the reduction in competitiveness with increasing dose, so that an optimum balance between sterility and competitiveness may be achieved. However if the irradiation procedure is not appropriately controlled the precise dose given will not be known accurately.

The Insect Pest Control sub-programme has been promoting the use of a radiochromic film dosimetry system **(Figure 19)** (Gafchromic ® film) and has produced a manual describing the calibration and use of the system that can be downloaded from our web site (http://www-naweb.iaea.org/nafa/ipc/public/d4_pbl_5_2.html). It was chosen to be relatively simple to use and cheap to buy, whilst providing adequate precision for an SIT programme. A number of SIT rearing facilities have been provided with the film, reader and dosimeter holders through IAEA Technical Cooperation projects. As an aid to the SIT programmes to assist them to determine if they are using the system correctly and achieving their target dose, a dosimetry inter-comparison service has been initiated. The service will send out a set of test and control dosimeters as a pair in a sealed pouch. The control dosimeters are separated from the test dosimeters, which are exposed in the programmes irradiator during their normal insect processing. On return to the laboratory the dosimeters will be read and the measured value compared to the target value. If a discrepancy is found the programme manager can adjust the irradiation protocol appropriately to bring the dose to the correct value. Any programme wishing to use the inter-comparison service should contact the sub-programme.

Figure 19. Dosimeter set.

[6] E2.05, activity 3

3.2. Fruit Fly Rearing and Quality Control

Extensive work has been carried out on two strains of Anastrepha fraterculus *originating from Peru and Argentina. Previous work had shown that there was a high degree of mating incompatibility between these strains in field cages. This was confirmed by studies in 2007 and the analysis was expanded to include studies on chromosomes, pheromones,* Wolbachia *and hybrids. The analysis revealed significant pre- and post-zygotic isolation, qualitative and quantitative differences in pheromone blends and chromosomal variation. These findings strongly support previous work that suggested that the nominal species* Anastrepha fraterculus *is indeed a species complex. The findings also have applied significance in relation to the development of regional SIT programmes against this pest.*

Activities on transgenic strains of fruit fly have moved from their development to their evaluation. During 2007 studies were carried out on classical genetic sexing strains carrying a fluorescent protein marker, a transgenic sexing strain and a transgenic strain that carries a mutation that kills embryos. The studies involved mass rearing, stability and field cage evaluation. In general all strains performed reasonably well and showed no major impact of the presence of the transgene. The sexing accuracy of the transgenic strain was very good and the males from the strain with the embryonic lethal completely sterilized females. These findings are very preliminary and they require much more robust testing before they can be integrated into SIT programmes.

The olive fruit fly Bactrocera oleae *is a pest of increasing significance in traditional countries of origin but has also invaded new areas. A long standing major constraint to the use of the SIT for this species has been the cost and unpredictability of the mass rearing process. The Unit is supporting an SIT feasibility study in Israel and is supplying sterile flies for field cage tests and eventually open field test releases. With the help of a consultant the rearing process has been greatly improved during 2007 and material is now being sent to many counterparts. Success in stabilizing and improving mass rearing will enable the Unit to play a unique role in SIT development for this species.*

A major investment was made in the fruit fly facilities in 2007 with the construction of a 250 m^2 climate controlled greenhouse. The greenhouse has been designed to allow the evaluation of transgenic strains under semi-field conditions. The construction is now complete and it is hoped that the environmental conditioning can be installed during 2008.

3.2.1. Improving Rearing Methodology for the Olive Fruit Fly *Bactrocera oleae* [7]

Reducing the cost and improving the efficiency of olive fly mass rearing is a pre-requisite to the use of the SIT for this species. The species is notoriously difficult to rear and colonies are frequently subject to fluctuation in numbers for unknown reasons. Considerable work has been done in the Unit in 2007 both for the conventional rearing and also for the use of a liquid diet for larval rearing. This is a very important project as the Unit will have to supply considerable numbers of sterile olive fruit flies to Israel in 2008.

3.2.1.1. Assessing liquid larval diet

The work was carried out during a consultancy by Dr. Stella Chang from the Agricultural Research Service (ARS) of the United States Department of Agriculture (USDA) in Hawaii. Olive fruit fly larvae are currently reared on a solid diet with cellulose as a bulking agent in addition to other ingredients, such as yeast-based products (brewer's yeast and soy protein), sugar, antimicrobial agents (nipagen, potassium benzoate), Tween 80, hydrochloric acid, and water. The objective of the work was to eliminate the high cost of cellulose by developing a liquid diet as has been done for other tephritid fruit flies.

Liquid larval diet is a novel cost effective way for fruit fly rearing and it has been successfully developed for medfly, oriental fruit fly, and melon fly in Hawaii and is in process of technology transfer to mass rearing facilities. This type of diet not only reduces the cost for spent diet management, but also releases storage space and labour. Furthermore, it can eliminate the potential pesticide contamination problem for bulking agents. The value of this diet lies in its liquid form and the fact that the the cellulose can be replaced with an inert sponge cloth.

Table 1. Liquid diet formulations

	Solid	Liquid						
Diet Ingredients	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Soy hydrolysate (g)	6	0	19	4	0	0	0	19
FNILS65 (ml)	0	19	0	0	4	5	4	0
Brewer's yeast(g)	15	0	57	12	0	0	0	57
LBI2240 (ml)	0	57	0	0	12	15	12	0
Potassium sorbate (g)	0.1	0.1	0.1	0.05	0.1	0.2	0.1	0.1
Nipagen (g)	0.4	0.4	0.4	0.2	0.2	0.2	0.2	0.4
Sugar (g)	4	4	4	2.75	2.75	12.81	2.75	4
Olive/wheat germ oil (ml)	4	4	4	2.75	2.75	2	2.75	4
Tween 80 (ml)	1.5	0	0	0	0	0	2	0
Water (ml)	110	160	160	100	100	100	100	320
HCL (ml)	0.9							
Citric acid (ml)		10	10	4	4	2.31	4	10
Cellulose (g)	55							

[7] E1.07, activity 5

Eight liquid diet formulations were tested and compared with the standard solid diet used in the Unit (**Table 1)**. The liquid diets excluded cellulose, Tween 80 and HCL. Due to the limited egg production from the colony, only 1000 eggs could be seeded to all eight diets. Most of them showed no or low recovery except diet #5. Diet #5 had an additional amount of soy hydrolysate and brewer's yeast as compared to the solid diet. However even with this diet the larvae survived only up to late 2nd instars and were unable to molt to the 3rd instar because there was no trace of the molts seen on the diet. Addition of Tween 80 and olive oil did not improve the situation. In the solid diet the olive oil is absorbed into the cellulose and other solid ingredients whereas in the liquid diet the oil floats on the surface, even with the addition of Tween 80. Thus in contrast to other tephritid species, liquid diet may be difficult to develop for the olive fruit fly. It is very likely that the microbial environment of the diet and inside the larvae plays a key role in ensuring optimal larval development.

3.2.1.2. Improving the solid larval diet

In November-December 2007, Dr. Ioannis Dimou, a consultant from the Laboratory of Applied Entomology, University of Crete, worked in the fruit fly group. He provided assistance in order to detect possible sources of contamination of the solid larval diet by fungi and bacteria. This is a major cause of poor larval survival.

The key aspects identified by Dr. Dimou to be improved included the redesign of adult cages, incubation of eggs after collection, seeding method, larval diet preparation and the final pH of the larval diet. These procedures in the rearing system could lead to contamination of larval diet and this, in turn, to low pupal recovery. A level-one testing of many larval trays revealed cases of microbial contamination (**Figure 20**) resulting in i) putrefaction/fermentation odor and darkening of color, which could be attributed to bacteria or yeasts and ii) dark mould patches, and spores of fungi probably belonging to *Aspergilus* sp. or *Rhizopus* sp. Similar types of contamination have been encountered also at the University of Crete's facilities. After this careful examination of the procedures, several important changes were made and were readily incorporated into the existing Seibersdorf rearing protocol. Olive fruit fly rearing is now stable and we are able to cope with the increasing demand from different laboratories and programmes.

Figure 20. Types of visible and odorous contamination encountered. Left: probably yeasts or bacteria, Right: mould.

3.2.1.3. Adult survival, egg production and egg hatch

Adult survival, egg production and egg hatch of the Valencia olive fruit fly strain was assessed. This strain originated from a cross between males from a wild strain from Valencia crossed with females from a long established laboratory strain. Three plexiglas cages were stacked one on top of the other (Cage 1 at the bottom, and Cage 3 at the top). The light intensity in each cage varied, with the top cage receiving the most light and the bottom cage receiving the least. Thirty five pairs of flies were placed in each cage and provided with food, water and egging substrates. Measurements were carried out over 23 days. Egg production was recorded daily, and dead insects removed and counted.

Hatch values ranged from 84.0% to 96.0%, with egg hatch increasing to a maximum of 96% on day 9 and then decreasing (**Figure 21**). Survival of females, on the other hand was slightly shorter in the Valencia strain, compared with the survival data from the Greek "Lab" colony held at the Laboratory of Applied Entomology, Crete. The flies in the cage with the highest light intensity appeared to have the lowest survival. **Figure 19** possibly indicates that females in Cage 3, which was the cage with higher light intensity, may be suffering a higher mortality. This however needs a replicated test for clarification.

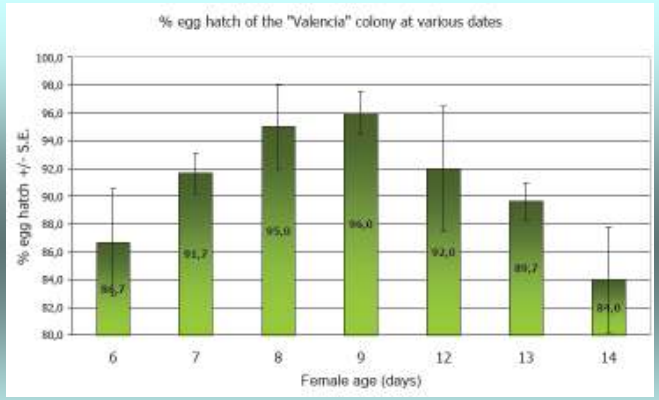


Figure 21. Mean percent egg hatch of the "Valencia" strain at various collection dates.

The number of eggs laid per alive female followed an initial "stabilization" period, after which individuals in the cage with the least light (Cage 1) appeared to gain a significant advantage in egg production. Again, a replicated test is needed here to follow up on this. Higher mortality in the cage with the most light (Cage 3) coupled with higher oviposition rate of females in Cage 1 finally result in an apparent higher total egg yield in Cage 1 (**Figure 22**). The parameter of most importance for efficient mass rearing is the total daily egg yield. The above results suggest that within the light intensity range of the specific rearing room there may be important effects on adult survival and fecundity, which can be taken advantage of to increase rearing efficiency.

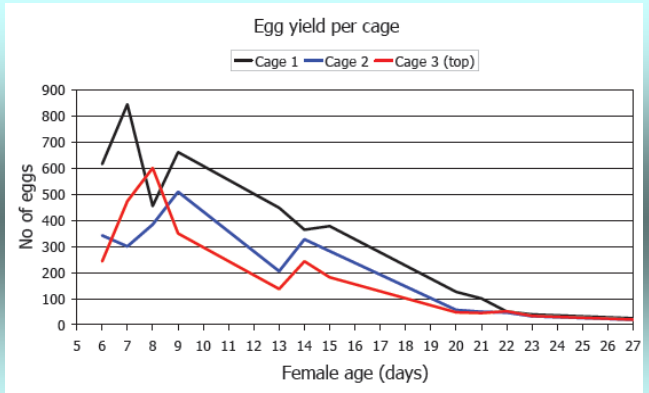


Figure 22. Daily egg yield in the three cages.

3.2.1.4. Low pH/Dish condition/Seeding method test

A larval bioassay was carried out to investigate the effect of pH on larval diet (4.8 in Seibersdorf versus 4.2 in Greece), the type of dish (new versus clean but used) and the egg seeding method (liquid seeding in Seibersdorf versus paper strip in Greece). These tests were carried out using a 2 x 2 x 2 factorial design with six replicates/treatment giving a total of 48 experimental units. Parameters measured were % egg-pupa recovery, pupal weight and % adult emergence. Appropriate numbers of eggs were measured by weighing on a precision scale. Dishes were examined for microbial contamination one week after seeding and valued on a 0-3 scale depending on the extent of visual contamination (0: no contamination, 1: small patches, 3: total medium surface covered). Pupae were weighed individually on the 5th or 6th day, after the initial water loss.

Out of a total of 48, only four dishes developed observable contamination and these were all following the liquid seeding method, with no effect of the other two factors (**Table 2**); this could indicate that egg seeding is advantageous for microbial growth. None of the three factors studied had a significant effect on the egg to pupal recovery, but a statistically significant effect of the seeding method on the weight of the pupae was detected, with paper (P) strip seeding method producing smaller pupae than the liquid (L) seeding method (**Figure 23**). Diet type and dish condition did not have any significant effect on the pupal weight. Seeding method had a statistically significant effect on the adult emergence, with paper strip seeding giving lower emergence values than liquid seeding **(Figure 24)**. Diet type and dish condition did not have an effect on the adult emergence.

Table 2. Microbial contamination in larval rearing dishes

PH	Dish	Seeding	Replicates	Contamination (0-3)	
				Fungal	Bacteria
Standard	New	Liquid	3	0	1
Standard	Washed	Liquid	5	0	1
Low	New	Liquid	2	0	2
Low	Washed	Liquid	2	0	1

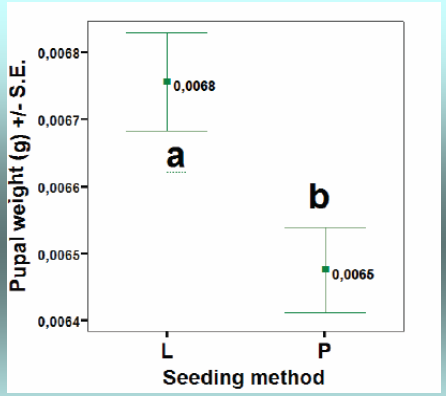


Figure 23. Pupal weight in relation to seeding method.

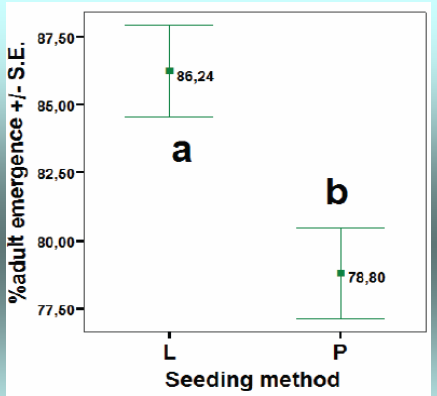


Figure 24. Adult emergence and seeding method.

The above results suggest that a diet with a lower and more appropriate pH around 3.9 – 4.0 can safely be used without any negative effects on the rearing parameters. However there were two instances where contamination developed even in this diet (**Table 2**), but this occurred in liquid seeded diets (which are subject to antimicrobial dilution and pH alteration). A low pH diet may therefore only be able to express its antimicrobial properties when seeded in a way that does not significantly dilute the antimicrobials or alter the pH. There has been no positive gain after using brand new larval dishes instead of reusing washed ones. Besides, using new dishes does not safeguard against contamination. Some results point out that paper strip egg seeding method might provide a good anti-contamination measure. However this practice had a significant negative effect on pupal weight and adult emergence, which cannot be overlooked. The mechanism of this effect is not clear, but it is possible that the strip is acting as a foreign-object obstruction inside the medium, impeding larval movement and/or feeding. If so, removal of the strip after the 1st instar might solve this problem.

3.2.1.5. Incubation duration test

This small test was stimulated by some of the previous experiments in which there was a significant increase in % recovery values in some replicates in which eggs were incubated for only 24h instead of 48h, the standard procedure.

The experiment looked at three incubation periods: 0 hour, 24h and 48h. Eggs were collected, counted to 100-egg batches under a stereoscopic microscope and seeded on the same and the two following days. Although pupal weight did not differ significantly between the three incubation periods, the % egg-pupa recovery was significantly lower after 0 hours of incubation than after 24h or 48h. There was a 4.8% recovery benefit of 24h incubation over 48h, but this difference did not turn out statistically significant (**Figure 25**).

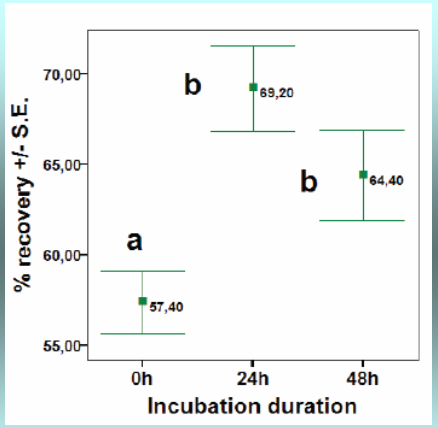


Figure 25. Egg-pupal recovery following three incubation temperatures.

The results of the trial show that fly quality as indicated by pupal weight is not affected by the length of the incubation period. Even 0 hour incubation can be used if necessary, with no decline in insect quality, but with a significant cost in % recovery. 24h incubation did not differ significantly from standard 48h incubation in % egg-pupa recovery, but the former gave higher recovery by almost 5%. The mean recovery values (**Figure 25**) suggest that a larger number of replications will most probably reveal that this benefit of 24h incubation in terms of % recovery is statistically significant.

3.2.2. Irradiation studies on an Israel-Greece Hybrid Strain of the Olive Fruit Fly (*Bactrocera oleae*) [8]

Ms. Esther Lavi, a scientific visitor from the Institute of Plant Protection, Agricultural Research Organization from Israel, conducted a series of radiation experiments in December 2007. She focused mainly in an Israel x Greece hybrid strain of this species, as this is the strain that will be used in 2008 for pilot field releases. However some experiments were carried out using the parental Greek laboratory strain.

The experiments involved the irradiation of olive fruit fly pupae at six different doses (20, 40, 60, 80, 100, 120 Gy) and following basic quality control tests (pupae weight, flight ability, percentage of pupae emergence, and sex ratio) and induction of sterility. Irradiation was given two days before emergence, and a group of non irradiated pupae from the same batch was used as control treatment. In the sterility induction experiments, irradiated flies were sorted by sex after emergence and placed in cages with non irradiated flies from the opposite sex. Flies were allowed to mate and lay eggs in egg-laying devices. Egg collection was followed for at least five days. Collected eggs were counted and incubated. Hatching percentage was determined 72h after egg collection.

[8] unplanned activity

The results showed that pupal weight, adult emergence rate and sex ratio, and flying ability for the two strains did not seem to be affected by the treatment the pupae received. **Figure 26** shows the data for the hybrid strain. When the response exhibited by the two strains was compared, non-significant differences were detected, suggesting that the differences in genetic background between these strains did not affect their response to gamma irradiation.

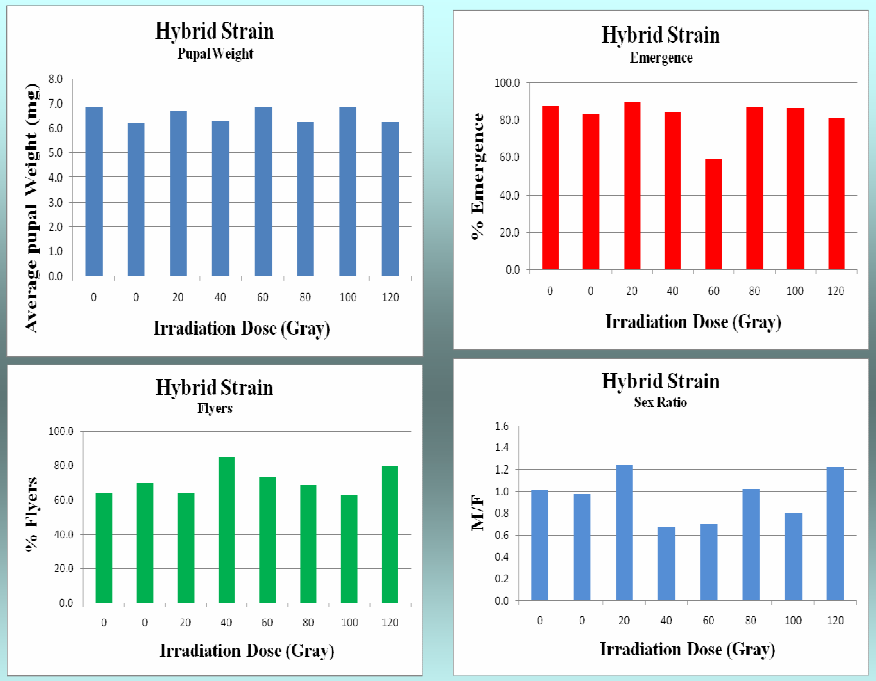


Figure 26. Effect of irradiation on pupal weight, % emergence, % flyers and sex ratio from the hybrid strain.

The results from the sterility induction experiments showed that, in general, both strains behaved similarly. When males were irradiated and females were fertile, there was a relatively high level of egg production. In contrast, when females were sterilized and males fertile, egg production dropped almost completely to zero after 20 Gy. Egg hatching with sterile males, on the other hand, dropped from 50% to less than 10% with increasing levels of irradiation. Of interest was to see that males irradiated with doses of 120 Gy were not fully sterile (**Figure 27**) and a low percentage of produced eggs by females still hatched (the viability of these hatched larvae was not investigated). Regarding sterilized females, egg hatching drops to zero after 20 Gy except in the hybrid line that showed a very low hatching of the produced eggs when irradiated with 60 Gy.

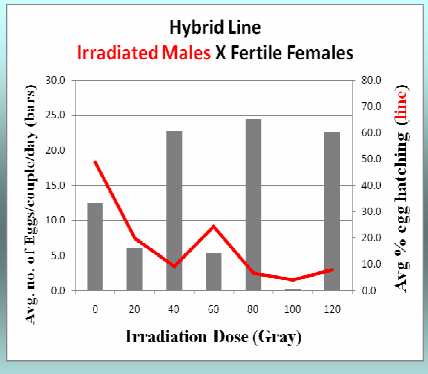


Figure 27. Egg production and egg hatch following irradiation of males of the hybrid line.

In summary, this preliminary study has shown that even low doses of sterilization (above 20 Gy), seem to completely induce sterility in female olive flies, without any noticeable effect on adult emergence, pupae weight, sex ratio or flying ability. High irradiation doses seem to have failed to completely induce sterility in male flies, although this aspect requires further investigation. Some data, especially those related to the induction of sterility in males with doses above 80 Gy, require to be verified.

3.2.3. Effect of Methoprene and Dietary Proteins on Behavior of Male Melon Flies (*Bactrocera cucurbitae*) [9]

3.2.3.1. Mating competitiveness in field cages

The effect of methoprene and protein on mating competitiveness of male melon flies was assessed in standard field cages at the Seibersdorf laboratories. The experiments consisted of four treatments: males treated with 1) methoprene (M) and protein (M+P+), 2) only protein (M-P+), 3) methoprene and sugar (M+P-), 4) sugar only (M-P-) and eight replicates were performed for each treatment. Twenty males of each treatment, identified by painting the thorax with a small spot of organic water based paint, were released together in the same field cage; 20 min later 20 mature females were released, i.e. 80 males competed for 20 females. Males 4, 5, 6, 7, 8 and 14 days old were used and the number of couples and mating duration were recorded. As in the previous experiments, addition of protein to the adult diet has a very significant effect on mating success of males when compared to males fed only sugar. However there was no effect of methoprene on fully mature males 13 to 16 days old with or without protein. Methoprene significantly accelerates male sexual maturity when protein is also provided. Males fed only on protein are sexually mature at age of 8 to 10 days; the addition of methoprene reduces the age of sexual maturity to day 5. If protein diet is not supplied, methoprene has no effect on sexual maturity. The results show that the age five to seven days is a period of induced sexual maturity.

The results (**Figure 28**) showed that males five to seven days old treated with methoprene and fed on protein out-competed males fed on protein but without methoprene, fed only methoprene and sugar of the same age and sugar fed fully mature males 14 days old. M+P+ males at days five and six have double the competitiveness of both M-P+ males of the same age and 14 day old M-P- males. At seven days of age the difference between M+P+ and M-P+ is reduced. The M-P+ males attained the same level of competitiveness as M+P+ males on day eight but M+P- and M-P- males perform very poorly. The males fed on protein with or without methoprene have similar mating competitiveness after eight days of age but the share of sugar fed males in mating competition was very low. The mating success of males fed on sugar and treated with methoprene remained negligible from day five to day eight.

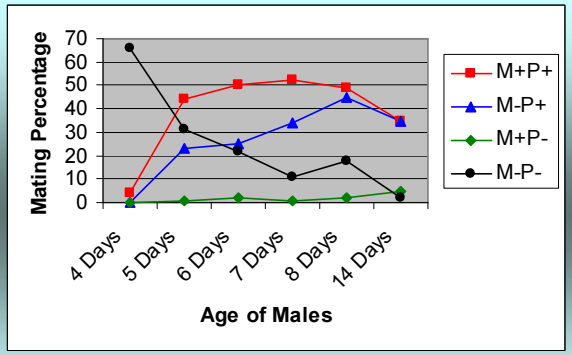


Figure 28. Mating success of *B. cucurbitae* males treated with methoprene and fed protein.

3.2.3.2. Longevity

The same treatments were used as above with five replicates and the observations were carried out in the laboratory. Twenty males from each treatment were released into 16 x 10 x 10 cm cage for each replication. Males were fed for 3, 4 or 5 days and then deprived of food and water. The dead males were removed every twelve hours. Within each feeding period there were very few differences between the treatments with perhaps the exception of the three day feeding period

[9] E1.06, activity 11

where M-P+ males appeared to die a little quicker than males from the other treatments. However there were differences between the feedings periods in terms of male survival. The longer the feeding period the quicker the flies died, For example for flies fed for five days and then deprived of food, 60% were dead after 48h. For flies fed only three days 40% of them died after 48h. This information is of course very important for SIT as it indicates what the best strategy should be in terms of treating the males before they are released.

3.2.3.3. Inhibition of female re-mating

The ability of sterile males to inhibit the re-mating of wild females is an important indicator for the success of SIT programme and this was evaluated in field cages using the same set of treatments as above. The females mated in the first mating were marked according to the male they mated with and given a normal adult protein diet for one day. They were then returned to the cage and exposed for re mating with all four types of males in competition. In the first mating and re-mating test the males (M+P+,M-P+,M+P-) were six days old having a competition against male (M-P-), 14 days old served as control. As reported in literature, *B. cucurbitae* females have a high tendency to remate. **Figure 29** shows data for the females that mated first with M+P+ males (first column) and the number of females re-mating with the four types of male is shown in the next four columns. There was no effect of any treatment on the inhibition of females to re-mate, but methoprene in combination with protein has higher percentage of mating with once mated female irrespective of the type of male in the first mating.

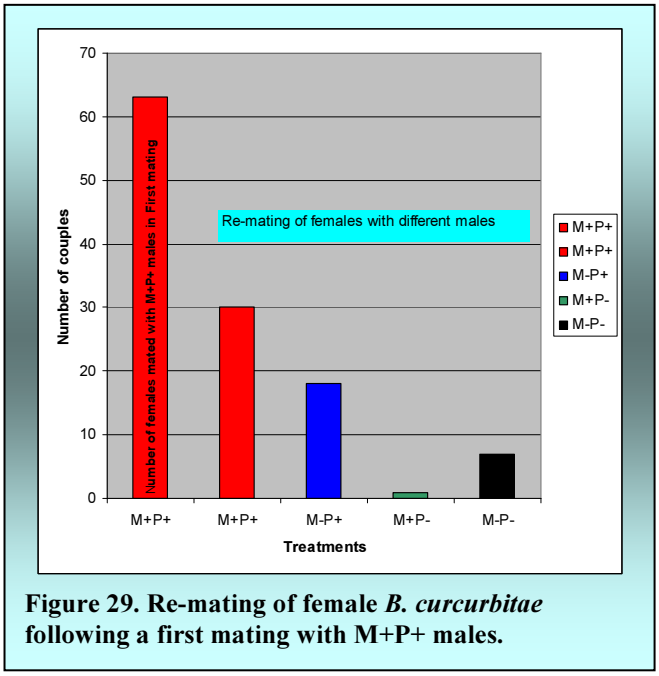


Figure 29. Re-mating of female *B. curcurbitae* following a first mating with M+P+ males.

3.2.4. Effect of Methoprene on the Mating Behavior of Two Strains of *Anastrepha fraterculus* [10]

Methoprene has been shown to reduce significantly the mean time of sexual maturation in *A. fraterculus*. Previous studies conducted by Mr. Diego Segura, consultant from INTA Castelar (Argentina), at the IAEA Laboratories at Seibersdorf showed that the methoprene treated males can compete under field cage conditions with untreated mature males. This was shown for males of two laboratory strains from Argentina and Peru. Some differences in the response of males to the treatment were detected between males from these two strains. However, these results needed to be confirmed given the relative low number of replicates of the Peru strain.

The effect of methoprene treatment on the sexual maturation of *A. fraterculus* females has never been addressed. Given the lack of a genetic sexing strain (GSS) for *A. fraterculus*, both males and females would be treated with methoprene before they are released into the open field. If methoprene induces acceleration in the female maturation process, then there is a threat that those

[10] E1.07, activity 4

females will mate with the sterile males, reducing the chances that these males find fertile females. On the other hand, if methoprene only accelerates sexual maturation in males, then this treatment could produce a similar effect as a GSS as the treatment would produce sterile males ready to mate and females that are still immature and will not interfere with those males.

To continue the evaluation of the sexual behavior and competitiveness of methoprene treated males under field cage conditions, Diego Segura visited our Laboratories during April and May 2007. His main objectives were: 1) to determine the existence of differences in the response to methoprene treatment between Argentina and Peru males; 2) to analyze the temporary increase in the sexual competitiveness of treated males in order to find the minimum age at which the males can be released, and 3) to assess the effect of methoprene treatment on female reproductive behavior. All the experiments were performed under field cage conditions at the FAO/IAEA Laboratories at Seibersdorf, following the standard procedure for field cage tests (FAO/IAEA/USDA 2003). Methoprene was delivered to the flies by applying 1µl of methoprene on the thorax after emergence. Flies were marked with a dot of water-based paint in their thorax prior release.

3.2.4.1. Differential response of Peru males to methoprene treatment

In 2006, the results were inconclusive as to whether methoprene induced early maturation in Peru males as was shown for Argentina males. **Figure 30** shows the mean percentage of matings obtained by each type of male in 2007. The results showed that 7 days-old treated males can compete with 10 days-old untreated males. In the control test, where 7 days-old males were not treated, 10 days-old males where more competitive as they obtained significantly more matings than the younger males. These results show that the treatment with methoprene allows seven day old Peru males to compete with mature males as was shown for Argentinean males in 2006.

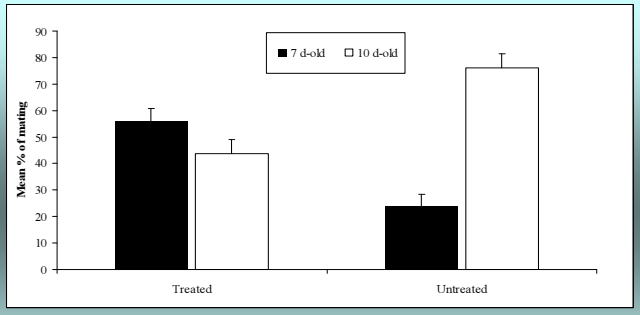


Figure 30. Matings achieved by each type of male (7 d-old methoprene treated or untreated, and 10 d-old untreated mature males) of the Peru population.

3.2.4.2. Competitiveness of males treated at different ages

Based on the sexual maturation curve described in laboratory studies in Argentina, males may be able to compete with mature untreated males even when they are younger than 7 days-old. This could allow the release of treated males before they are 7 days-old. To obtain information about the minimum age at which males become sexually competitive, field cage tests were performed releasing 5, 6 or 7 days-old methoprene treated males versus 10 days-old untreated males. **Figure 31** shows the percentage of mating obtained by each type

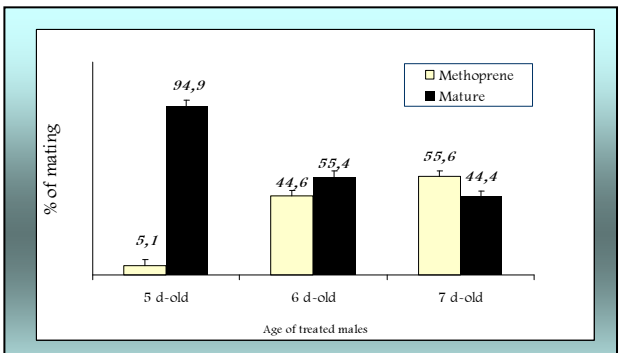


Figure 31. Matings obtained by each type of male (5, 6 or 7 days-old treated males vs. 10 days-old mature males) from the Argentina population.

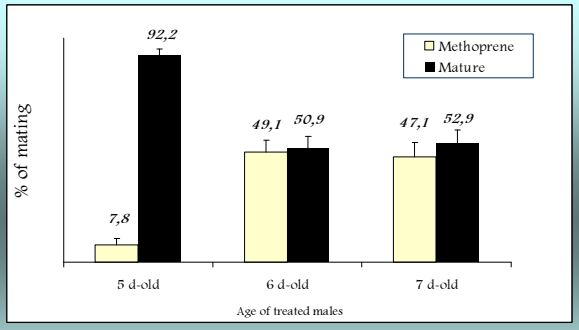


Figure 32. Matings obtained by each type of male (5, 6 or 7 days-old treated males vs. 10 days-old mature males) from the Peru population.

of treated males for the Argentina strain. Five days-old males were engaged only in 5% of all the matings whilst 6 and 7 days-old treated males proved to be as competitive as mature males, obtaining even more couples than the mature males in some cases. The percentage of mating obtained by each type of treated males from Peru is shown in **Figure 32**. As with males from Argentina, 5 days-old treated males were unable to compete with mature males (7.8% of the matings). After they were 6 days-old, the percentage of mating obtained by the treated males raised to 50%, very similar to what was found for 7 days-old males. These results indicate that the treatment with methoprene produced competitive males six days after emergence. This phenomenon was found for males of the two strains under study.

3.2.4.3. Effect on female reproductive behavior

In order to analyze if the methoprene treatment also has an effect on the sexual maturation of the females, 7 days-old treated females and males were released inside a field cage together with 10 days-old untreated males and 14 days-old untreated females. As a control, the same experiment was performed replacing the 7 days-old treated females, with 7 days-old untreated ones.

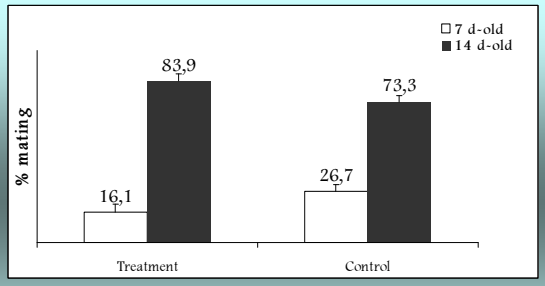


Figure 33. Mating involving different types of females for the Argentina population.

The percentage of mating obtained by each type of female is shown in **Figure 33** for the Argentina strain, and in **Figure 34** for the Peru strain. For both strains, the mature females obtained most of the matings (around 80%), irrespective of whether the 7 days old females had been treated with methoprene or not. Although, the % mating obtained by 7 days-old females was slightly lower in the treatment experiment (especially for Argentina), there were no statistical differences between treatments in either of the two strains (ANOVA for Argentina: $F = 3.76$; $p = 0.08$; ANOVA for Perú: $F = O.84$; $p = 0.38$).

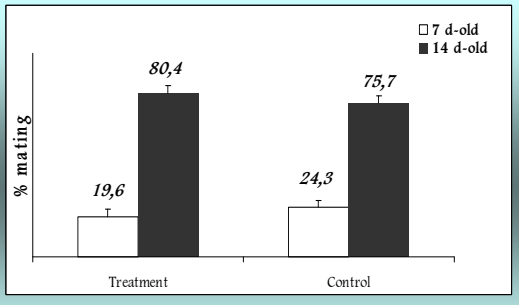


Figure 34. Mating involving different types of females for the Peru population.

These results have important implications to support the treatment with methoprene. Since no GSS has been developed for *A. fraterculus*, sterile methoprene treated males would be released along with females, which will be also treated with methoprene. If the treatment causes, as an unwanted result, females to start to mate at the same time as the released males, they could reduce the impact of SIT, since the

sterile males will hardly find fertile females. However, the present results suggest that the JH treatment does not accelerate female maturation, and that the treatment induces a separation of the sexes regarding their readiness to mate. This could be envisaged as a "sexing effect" of the methoprene, given that the final result is the presence in the field of sterile males ready to mate and sterile females that are not yet ready, and will allow the males to look for fertile females, which is similar to release only males, as in a SIT programme based on a GSS. Nonetheless, we found that a considerable amount of females were ready to mate when they were only 7 days-old (ca. 20%). Future studies should be directed to find a pre-release treatment to reduce this percentage.

3.2.5. Mass Rearing of a Medfly Transgenic Sexing Strain [11]

To evaluate the potential of the transgenic sexing strain of medfly OX3376B, developed by Oxitec Limited, for mass-rearing and for use in an SIT control programme, Dr. Neil Morrison from Oxitec visited our laboratories with the aim of analyzing several parameters that contribute to productivity and comparing them with those of VIENNA-8, a temperature-sensitive lethal (*tsl*) sexing strain. The OX3376B strain carries a female-lethal transgene insertion. The lethal effect of the transgene insertion is repressed in the presence of tetracycline or related compounds (as chlortetracycline, CTC) in the larval diet. The strain is homozygous for the insertion and marked with a green fluorescent protein. Both strains were followed for six generations of mass rearing, in which the amount of CTC added to the larval diet of OX3376B, adult diet and drinking water increased from 0 ug/mg (or ml) to 200 ug/mg (or ml). The stability of the OX3376B transgene insertion was also examined by assessment of marker expression and efficient sexing in each generation. The ability of OX3376B males to compete with wild-type males for mates was also studied.

3.2.5.1. Egg production

Egg production in each mass-rearing cage was initially encouraging (**Figure 35**), but the colony took three generations to reach a level where the cage was able to be stocked with a volume of pupae that provided mass-rearing adult density. This was due to the sub-optimal amount of CTC used in the larval diet and adult drinking water as well. After increasing the CTC concentration in larval diet to 100 ug/ml, in the fourth generation (Cage 4) egg yield increased to 193 and 202 eggs/female in cage 5 **(Figure 35)**.

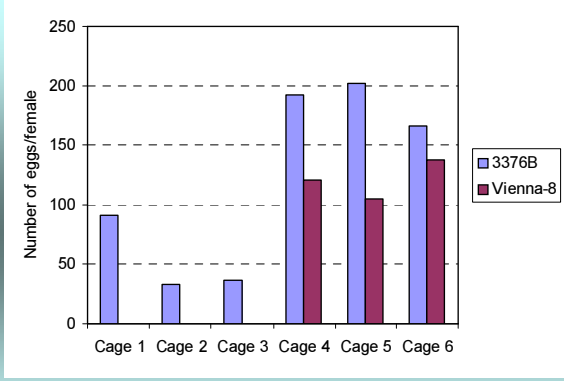


Figure 35. Eggs/female collected from the mass-rearing cages of OX3376B and Vienna-8 strains.

Looking at total eggs collected from cages containing standard mass-rearing density of adults (1600 ml pupae), there was some variation in yield between OX3376B cages (**Figure 36**). In addition, total eggs collected from OX3376B cages were consistently lower than from VIENNA-8

[11] E1.06, activity 10

cages. Conversely, OX3376B egg production in the first six days was, on average, higher than that of VIENNA-8.

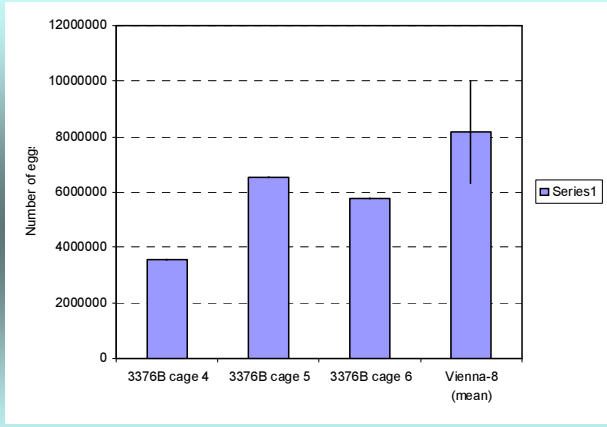


Figure 36. Total number of eggs collected from mass-rearing cages of OX3376B and Vienna-8 strains.

The lower egg yield in OX3376B is most probably due to two factors. Firstly, VIENNA-8 adults survive for longer than their OX3376B counterparts, and so can lay eggs for a longer period. The second factor that may have contributed to the higher egg yield of VIENNA-8 compared with OX3376B is that the relative numbers of males and females can be controlled by selecting batches of pupae with different proportions of brown and white pupae. OX3376B, however, cannot be manipulated in such a way.

3.2.5.2. Egg hatch

Eggs were collected for 12 days and assessed for hatch (**Figure 37**). This experiment indicated that the hatch rate of OX3376B eggs (69% or higher) remains comparable with those of VIENNA-8 for around nine days, but drops thereafter to below 10% on day 12. VIENNA-8 egg hatch rate remains at 67% or higher throughout this period. These data imply that, after 10 days of egg-laying, the productivity of OX3376B cages is reduced by a reduced egg hatch rate and probably also due to the death of female adults.

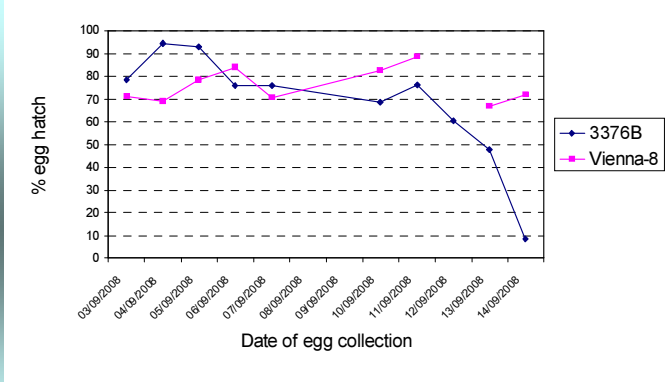


Figure 37. Egg hatch rate of samples collected during 12 days, starting on the first day of laying.

3.2.5.3. Egg survival to adult

From samples of 1000 eggs, the egg-to-adult survival was measured in the 6th generation (**Figure 38**). Optimum survival (63-68%) was found in OX3376B eggs placed on diet with 100-200 ug/ml CTC. On diet with no CTC, OX3376B survival was reduced by approximately half (due to female lethality). Survival of VIENNA-8 eggs to adulthood was found to be lower in these experiments (42.6%) due to the translocation. Further work with OX3376B will indicate whether this is a consistent finding.

When the proportion of males and females was analyzed, OX3376B pupae reared on larval diet with CTC concentrations of 100-200 ug/ml produce the highest proportion of females (43-46%).

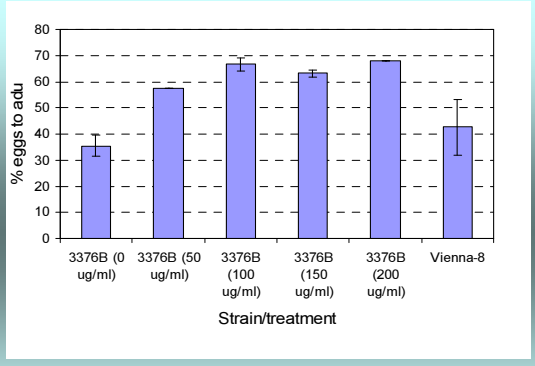


Figure 38. Percentage egg-to-adult survival on different concentrations of CTC, compared with that of VIENNA-8 eggs.

In diet with 50 ug/ml CTC, female emergence suffers, with 39% of pupae emerging into females. On all of these CTC concentrations, the proportion of males remains fairly constant (46-50%). From VIENNA-8 pupae, emerging males also outnumber females (44% and 40%, respectively). Significantly, no females emerged from OX3376B pupae which were reared as larvae in the absence of CTC; 1599 males emerged from 1767 pupae.

3.2.5.4. Pupal production during mass rearing

The volume of pupae collected was highest from OX3376B on 100-200 ug/ml CTC diet (**Figure 39**). The mean production of VIENNA-8 was markedly lower than those of 100 and 200 ug/ml CTC OX3376B, although there was high variation in these data and further repeats are needed. The yield of OX3376B pupae from 50 ug/ml CTC diet was less than half of that from the higher CTC concentrations. It is not clear why the volume of pupae in the non heat treated VIENNA is so low.

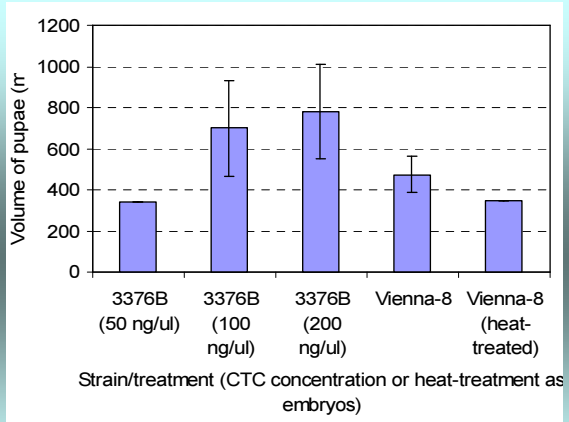


Figure 39. Mean yields of pupae (ml pupae/diet tray) from different treatments with OX3376B and Vienna-8.

3.2.5.5. CTC dosimetry on mass rearing diets

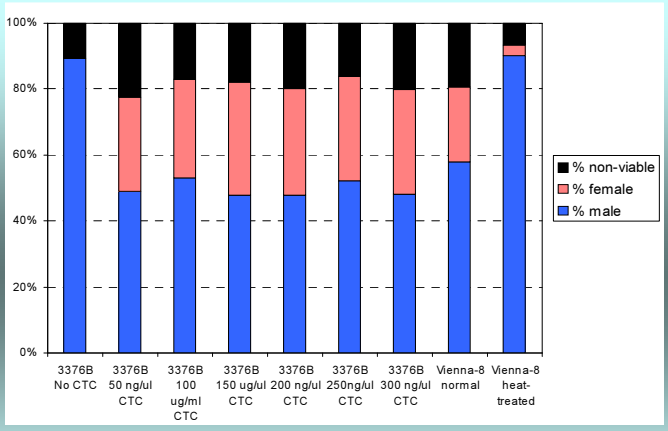


Figure 40. Emergence of pupae samples taken in the first 5 days of pupation from OX3376B reared on different CTC concentrations and from Vienna-8, showing proportions of males, females and non-viable pupae/adults.

No females were produced from OX3376B pupae, reared as larvae on a diet with no CTC. However, there was also some female lethality in OX3376B pupae reared as larvae on CTC containing diet (**Figure 40**). At higher CTC concentrations, the proportion of males and females remains fairly stable. Fewer females (mean, 32%) than males (mean, 50%) are produced. With VIENNA-8, however, 58% of the pupae emerge as

males and 23% as females. This indicates some females die as larvae, which may be due to overheating in the diet trays. It is possible that with better ventilation loss of females can be reduced. From VIENNA-8 eggs that were heat-treated at 34ºC for 24 hours female survival was 3%.

3.2.5.6. Transgene stability

To analyse the presence of the OX3376B transgene (and expression of the marker), a minimum of 500 pupae were examined at each generation. No wild-type pupae were found at any stage. The brightness of the green fluorescent marker was not high and therefore not easy to work with, especially in late pupae. A quicker method of screening was devised, in which pupae were crushed and their soft tissues screened for fluorescence. This method improved screening efficiency but would not have allowed out-crossing, and examination of progeny of any potential wild-types (non-fluorescent individuals) found. Another method of screening for OX3376B transgene instability is to rear the strain in the absence of tetracycline and assess female-specific lethality in a large number of individuals. By rearing OX3376B embryos off CTC on mass-rearing diet, it was possible to collect and screen over 6000 pupae for GFP fluorescence, with no instances of female adults being identified. These data provided strong evidence for high stability of both marker expression and female-specific lethality off CTC.

3.2.5.7. Mating competition experiments

Mating competitiveness tests were carried out in the greenhouses of the IAEA Entomology Unit in Seibersdorf. Males from the transgenic OX3376B strains had to compete with wild-type males, for wild-type females. Following the standard procedure for mating competitiveness tests, it was found that, although not as competitive as the Argentina males, the OX3376B males did reach the generally minimum accepted percentage of mating to be achieved by sterile males according to the standard set by FAO/IAEA/USDA (2003). Tests are currently being carried out to include a comparison of OX3376B males and VIENNA-8 males competing with each other and with wild-type males in different experiments.

3.2.6. Mating Competitiveness of Genetic Sexing Strains with a Fluorescent Marker [12]

Two different VIENNA-8 genetic sexing strains, VIENNA-8/2 and VIENNA-8/4 that carry different transgene insertions expressing red fluorescent protein either during the pupal or adult stage were compared with non-transgenic VIENNA-8 and with wild flies from Kawai Island in Hawaii or laboratory adapted wild males from Argentina. Fluorescent protein expression could potentially be used to replace the conventional sterile insect marking by fluorescent powder to identify sterile insects in the field. The transgenic strains were described in the **Activities Report 2005** (where VIENNA-8/2 refers to strain 7-4M5f which has a very high level of fluorescence in all developmental stages. VIENNA-8/4 refers to strain 2-3M2m which has a low and variable expression of fluorescence). These strains were chosen to assess if the level of fluorescence affected competitiveness. Mating compatibility tests were performed at the Entomology Unit in Seibersdorf, following appropriate quarantine protocols to avoid any insect escape.

Males from each transgenic VIENNA-8 strain competed against males from the non-transgenic VIENNA-8 strain and males from a wild strain, either from Argentina or Hawaii. On the day of the test, 20 sexually mature virgin males from each of the three tested strains were released into

[12] E1.06, activity 10

the cage around 08:30 and about 15-20 min later, 20 virgin sexually mature wild females were released. Mating pairs were collected as they formed by allowing the pair to walk into a small vial and the strains identified. Proportion of Mating (PM) and the proportion of males that participated in mating were calculated based on the number and type of mating recorded for the wild and laboratory flies. Mating compatibility and competitiveness for each experiment was determined by means of the Relative Sterility Index (RSI). In all field cage tests the PM index was high indicating that the conditions in the cage were very suitable for normal mating behaviour resulting in a high degree of sexual activity during the test period.

The RSI values for all the strains are shown in **Figure 41**. It is clear that transgenic GSS have a similar level of competitiveness as the non-transgenic GSS. However, as expected the competitiveness with wild strain from Hawaii was lower than that with Argentina as the latter strain was already somewhat adapted to laboratory rearing. As it seems that sexual behaviour of the transgenic is comparable to VIENNA-8 GSS, further studies should be conducted to determiner whether or not the fluorescent protein is expressed after exposure to the field and trapping conditions in order to see if fluorescent strains can be released into the field without dye marking.

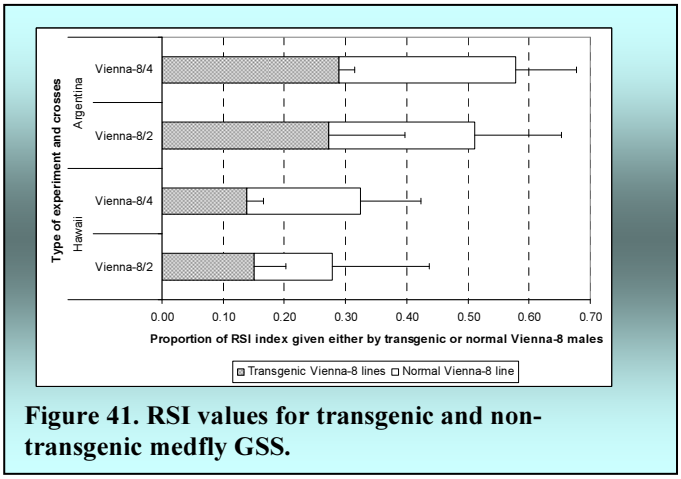


Figure 41. RSI values for transgenic and non-transgenic medfly GSS.

3.2.7. Mating Competitiveness of a Transgenic Strain with an Early Embryonic Lethal [13]

Conditional expression of early embryonic dominant lethal genes can provide an alternative to the use of radiation induced dominant lethal mutations as a means of inducing sterility in target pest populations. Marc Schetelig from the Georg-August-University, Goettingen, has developed such strains in medfly which are also marked with a red fluorescent protein. The mating competitiveness of one of these lines, #67, was assessed in field cages when competing against wild flies from Argentina. When males from the transgenic strain mate with wild-type females the embryos die in the absence of tetracycline. Mating compatibility tests were performed at the Entomology Unit in Seibersdorf, and followed appropriate quarantine protocols to avoid any insect escape.

Males from line #67, either irradiated (140 Gy, 48h before emergence), or unirradiated competed with unirradiated wild type Argentina males for unirradiated wild type females. Mating compatibility tests were carried out in a screened square cage inside a greenhouse. On the day of the test, 20 sexually mature unirradiated Argentina males, 20 unirradiated and 20 irradiated males from line #67 were released into the cage around 8:30. Approximately 15-20 min later, 20 virgin and sexually mature Argentina females were released. The type of mating couple was recorded and 12 replicates were carried out. PM and the proportion of males that participated in mating were calculated based on the number and type of mating recorded. After the mating couples

[13] E1.06, activity 7

separated females were separated into groups, depending on male partner, and transferred to small egging cages in order to determine their fertility.

Results have shown that in all replicates the PM index was 43 ± 5 indicating that there was an acceptable degree of sexual activity during the test period. Males of the transgenic line #67 competed very well, both when irradiated and unirradiated (**Figure 42**). However, irradiation did reduce the competitiveness somewhat. The % hatch of eggs oviposited by females mated by the different males in the field cage is shown in **Table 3**. As expected irradiated males from line #67 were fully sterile and the wild-type matings were fully fertile. The embryonic lethal transgene in the absence of tetracycline caused complete sterility in females mated with unirradiated males from line #67. Larger studies comparing line #67 with wild strains directly collected from the field should be conducted to corroborate these preliminary results.

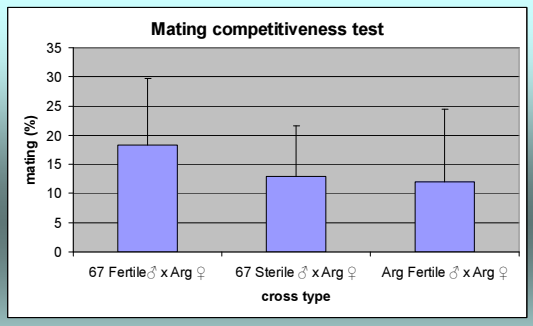


Figure 42. Proportion of mating of each type of male, 20 males of each type were competing for 20 Argentinean females.

Table 3. Egg hatch from mated females

Male	Female	No. of Eggs	No. Hatched	% Hatch
#67 Unirrad.	Arg.	775	0	0
#67 Irrad	Arg.	887	0	0
Arg. Unirrad	Arg.	821	740	90.13

3.2.8. Isotopic Method to Distinguish Between Wild and Laboratory Flies [14]

Monitoring of sterile to wild insect ratios is essential to follow the progress in SIT programmes. The main methods used for marking fruit flies are dyes in the diet or fluorescent dusts. In some instances there may be doubt as to the presence of a marker. Another issue especially from a legal perspective is the risk of an unintentional release of fertile un-marked factory-reared flies. Having a factory-specific intrinsic marker overcomes some of these difficulties and complements existent marking technologies. Fortunately nature itself provides us with an elegant marker. Mass-reared Mediterranean fruit flies are usually reared on a sugar rich diet and in most tropical countries the source of sugar in the factory diet is sugar cane. Sugar cane has a different photosynthetic pathway to most other commercial crops, in particular fruit crops. This photosynthetic difference leads to a distinctive stable isotope signature of the cane sugar; this isotopic signature is conserved in the mass-reared flies and can be easily detected using an elemental analyzer linked to an isotope ratio mass spectrometer (EA-IRMS). This may sound a complex procedure; however it is a simple case of collecting the fruit fly and placing it in a small tin capsule, drying it and then running it on the EA-IRMS. A sample typically takes 5-10 minutes to analyze and the procedure is fully automated. This isotopic analysis is available on a contract basis from a number of semi-commercial laboratories and commonly costs US $5-25 per sample.

A consultant, Ms. Rebecca Hood, has been evaluating the presence and persistence of the isotopic marker in Mediterranean fruit flies. Field and mass-reared flies have been collected from around

[14] unplanned activity

the world to determine whether there is a distinctive isotopic difference in sugar cane mass-reared flies and wild flies. Additional experiments have been conducted to determine whether the isotopic signature of mass-reared flies is persistent throughout the lifetime of released Mediterranean fruit flies. It was shown that it is indeed possible to use the isotopic signature of mass-reared flies to distinguish them from wild populations **(Figure 43)**. It was also shown that the isotopic signature persists long enough in the adult fly to confidently distinguish mass-reared flies from wild flies. The beauty of this method is that SIT factory managers do not need to change their production practices in any way, but when there is doubt about the origin of a particular fly this method could be used for confirmation of origin without further method development. Another advantage of this technique is that it can be used as an intrinsic marker of any factory-reared insect species using a sugar-based diet. Given the simplicity and the robustness of the method, factories currently using sugar beet sugar, which does not have a distinctively different isotopic signature to most commercial crops, may consider switching to a sugar cane source. In most cases this would not confer significant increases in production costs but would provide them with a simple factory marker. A paper detailing these experiments is in preparation.

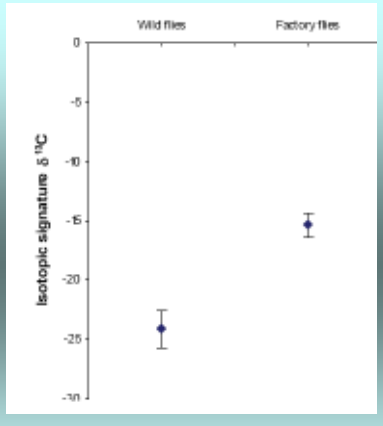


Figure 43. The isotopic signature of wild and mass-reared Mediterranean fruit flies.

Further experiments have shown that the marker is persistent throughout the normal life span of the fly and that it is possible to distinguish factory reared from native flies with greater than 99% certainty. (**Figure 44**)

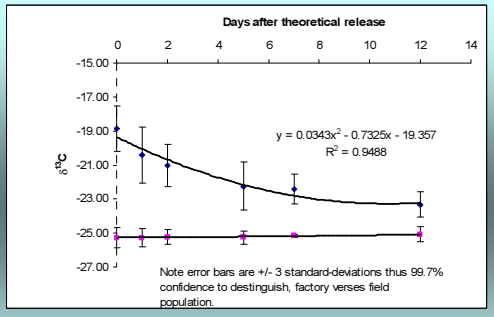


Figure 44. The isotopic signature created by the factory diet is conserved over the lifespan of the fly.

This information combined with other additional studies, which have shown it is possible to directly measure the isotopic signature of the spermathecae of the fruit fly, provides an opportunity to develop a method to estimate sterile insect insemination rates in the field. This technique is simple, cheap, robust and could possibly be used for a wide range of fruit flies in SIT programmes without the extensive development work associated with molecular based techniques.

3.2.9. Studies on Strains of *Anastrepha fraterculus* from Argentina and Peru [15]

Previous studies **(Activities Reports 2005 and 2006)** showed high levels of pre-zygotic isolation between two laboratory strains from Argentina and Peru. To further analyze this observation a series of experiments was carried out on these strains and on the reciprocal hybrids between them. This included mating compatibility studies in field cages, viability and fecundity studies in the

[15] unplanned activity

laboratory, biochemical analysis of male sexual pheromones, analysis of mitotic and polytene chromosomes and genotyping of *Wolbachia* infections. The two strains from Argentina and Peru have been maintained in the laboratory under identical rearing conditions since 2002.

3.2.9.1. Pre-zygotic isolation

The levels of pre-zygotic isolation were high for bisexual and unisexual tests for the parental strains as shown by the significantly high Index of Sexual Isolation (ISI) values **(Table 4)**. Although there was a tendency for lower ISI values in the unisexual tests with Argentina females, compared to the tests with Peru females, the differences were not statistically significant (t = 1.911; df = 14; P = 0.078). For hybrids, the level of pre-zygotic isolation was reduced **(Table 4)**. However, with Argentina males and H_{AP} hybrids, Argentina females still significantly preferred Argentina males in two replicates ($\chi^2 > 3.83$; $P < 0.05$) but the five replicates were homogeneous ($\chi^2 = 4.93$; df = 4; $P > 0.05$).

Table 4. Mating (%) and sexual compatibility in bisex(ual) and unisex (ual) field cage tests for two populations of *A. fraterculus* from Peru (P) and Argentina (A) and their F_1 hybrids

Test	Males		Females		ISI	c^2
Bisex	**A**	**P**	**A**	**P**	0.77 ± 0.05	142.74***
Unisex A	**A**	**P**	**A**		0.73 ± 0.05	76.96***
Unisex P	**A**	**P**	**P**		0.86 ± 0.04	88.66***
Unisex H_{AP}–A	**A**	**H_{AP}**	**A**		0.30 ± 0.12	6.70 **
Unisex H_{PA}–A	**A**	**H_{PA}**	**A**		0.15 ± 0.11	1.17
Unisex H_{AP}–P	**P**	**H_{AP}**	**P**		0.10 ± 0.10	0.67
Unisex H_{PA}–P	**P**	**H_{PA}**	**P**		0.13 ± 0.09	1.80

H_{AP} = F_1 hybrid from matings between Argentina males and Peru females
H_{PA} = F_1 hybrid from matings between Peru males and Argentina females

3.2.9.2. Post-zygotic isolation

All crosses involving the parental strains and their F1 hybrids produced viable progeny and in all crosses adult emergence was high. In four crosses, Arg x Peru, H_{PA} x H_{AP}, Arg x H_{AP} and H_{PA} x Peru, there was a significant reduction in egg hatch combined with a reduced larval viability (**Table 5**). The reduced egg hatch is restricted to specific crosses where the males are either from the Peru strain or H_{AP} hybrids (i.e. males from a cross between Peru females and Argentina males suggesting a possible maternal effect of Peru females) and the females are either from the Argentina strain or H_{PA} hybrids (i.e. females from a cross between Argentina females and Peru males (H_{PA}). Furthermore, one of these crosses, H_{PA} x H_{AP}, in addition to the

Table 5. Egg hatch, egg-pupal survival, and sex ratios in matings between Peru (P) and Arg (A) and F_1 hybrids

Cross F x M	% Egg Hatch	% Eggs to Pupae	Sex Ratio F/M
P x A	81 ± 6b	70 ± 5a	0.98 ± 0.04c
A x P	27 ± 3c	22 ± 3d	1.02 ± 0.32c
A x A	83 ± 6b	75 ± 3a	0.97 ± 0.17c
P x P	80 ± 3b	72 ± 4a	0.99 ± 0.12c
H_{PA} x H_{PA}	78 ± 5b	68 ± 4a	1.01 ± 0.20c
H_{PA} x H_{AP}	47 ± 6d	36 ± 6c	1.64 ± 0.20b
H_{AP} x H_{PA}	80 ± 4b	64 ± 21a	0.98 ± 0.10c
H_{AP} x H_{AP}	72 ± 9b	65 ± 9a	0.74 ± 0.10d
A x H_{PA}	79 ± 6b	67 ± 12a	0.97 ± 0.01c
A x H_{AP}	55 ± 6d	50 ± 7bc	0.88 ± 0.01d
P x H_{PA}	92 ± 4a	81 ± 14a	1.00 ± 0.20c
P x H_{AP}	85 ± 3ab	67 ± 21a	1.03 ± 0.10c
H_{PA} x A	74 ± 1b	50 ± 22b	2.33 ± 0.20a
H_{AP} x A	89 ± 2ab	78 ± 15a	0.93 ± 0.01d
H_{PA} x P	27 ± 3e	20 ± 4d	1.00 ± 0.20c
H_{AP} x P	70 ± 5b	62 ± 9b	0.86 ± 0.10d

low viability, shows a sex ratio distortion in favor of females. A similar sex ratio distortion was observed in an additional cross, H_{PA} x Arg, although this was not associated with a significant reduction in egg hatch. However, the progeny in these last two crosses have two common characteristics; they have the cytoplasm of the Argentina strain whilst the male sex chromosome complement is expected to be the same (X^AY^A, X^PY^A).

3.2.9.3. Mitotic chromosomes

A. fraterculus has six pairs of acrocentric chromosomes, including one pair of highly heterochromatic sex chromosomes with the male being XY. However, the strains from Argentina and Peru can be differentiated by the size and the morphology of sex chromosomes following Giemsa staining and C-banding. The Argentina strain **(Figure 45)** has a large X chromosome with two prominent C-bands located at the two tips, the one being larger than the other. The Y chromosome is smaller than the X and exhibits also two C-bands, the first on the proximal tip and the other in the sub median region. In the Peru strain, both X and Y chromosomes are large and similar in size **(Figure 46)**.

Figure 45 Argentina mitotic chromosomes.

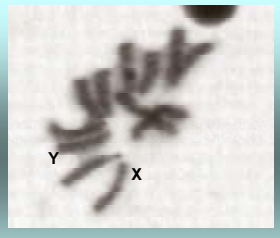


Figure 46. Peru mitotic chromosomes.

3.2.9.4. Polytene chromosomes

The polytene complement consists of five long chromosomes, probably corresponding to the five autosomes of the mitotic nuclei. Sex chromosomes, since they are highly heterochromatic, are not expected to form polytene elements as in all Tephritid species so far analyzed. There is a substantial similarity in the banding pattern between the two parental strains, especially at the chromosome ends making it difficult to differentiate between the two strains. However, in contrast to the Argentina strain, the Peru strain showed significant chromosomal polymorphisms, as shown in **Figure 47** where three different chromosome rearrangements are indicated.

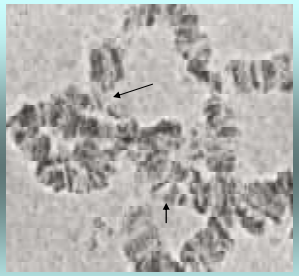


Figure 47. Peru polytene chromosomes.

F_1 hybrids showed extensive asynapsis **(Figure 48)** along the whole chromosome complement and this asynapsis is observed in almost all chromosome ends in spite of significant similarities in banding patterns. The degree of asynapsis in progeny of inbred F_1 flies was variable, probably due to the segregation and independent assortment of homologous chromosomes. However, there are cases where a whole chromosome or a significant part exhibits complete synapsis.

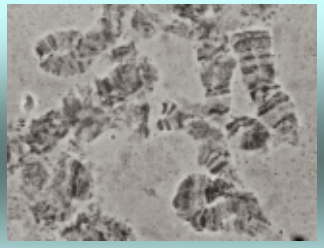


Figure 48. F1 hybrids showing asynapsis.

3.2.9.5. Pheromone analysis

There were significant qualitative and quantitative differences in volatiles released by Argentina and Peru males. Argentina males produced small amounts of (E)-β-ocimene, nonanal and larger amounts of (Z)-3-nonen-1-ol, benzoic acid, suspensolide, (Z,E)-α-farnesene, (E,E)-α-farnesene, anastrephin and epianastrephin **(Figure 49)** but no detectable amounts (E)-α-bergamontene or β-bisaboline. Peru males produced small amounts of limonene, nonanal and (Z)-3-nonen-1-ol along with relatively large amounts of ocimene, (E)-α-bergamotene, (E.E)-α-farnesene and β-bisaboline along with suspensolide, anastrephin and epianastrephin but no (Z,E)-α-farnesene was detected (**Figure 49**). Volatiles obtained from F_1 hybrid males from reciprocal crosses were surprisingly similar in both the number and ratios of compounds released. All collections from both hybrid males contained all of the compounds identified in both parental strains. No statistical differences in average amounts of compounds released per hour or in ratios of compounds were found.

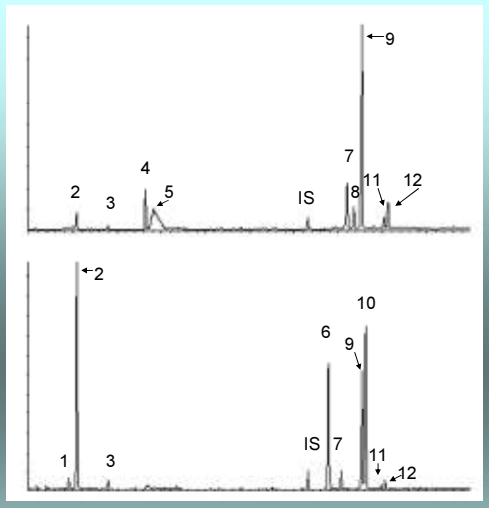


Figure 49. Comparisons of total ion chromatograms (EI spectra) obtained from analysis of volatiles collected from groups of males of the Argentina (upper) and Peru (lower) strain. **Compounds are 1 limonene, 2 (E)-β-ocimene, 3 (Z)-nonanal, 4 (Z)-3-nonenol, 5 benzoic acid, IS - internal standard, 6 (E)-α-bergamontene, 7 suspensolide, 8 (Z,E)-α-farnesene, 9 (E,E)-α-farnesene, 10 β-bisaboline, 11 anastrephin, 12 epianastrephin.**

3.2.9.6. Wolbachia

All strains tested were infected with *Wolbachia* based on the *wsp*-specific PCR assay (Zhou et al. 1998) and *AluI*-based RFLP analysis suggested that strains from Piracicaba, Argentina and Peru are probably singly infected while a strain of unknown origin is probably double-infected. The singly infected populations carry a *Wolbachia* strain which is identical to wSpt (A *Wolbachia* supergroup) naturally infecting *Drosophila septentriosaltans*. The only difference observed between the three strains is a conserved substitution in position 658 of the *wsp* gene of *Wolbachia* in the Argentina strain. The doubly infected strain is infected with an A-supergroup *Wolbachia* strain identical to *w*AlbA naturally infecting the mosquito *Aedes albopictus* and a B-supergroup strain closely related to the *w*Ma strain naturally infecting *Drosophila mauritiana*.

3.2.9.7. Conclusions

Major differences have been demonstrated at the behavioral, biochemical, cytological and genetic level between two strains of *A. fraterculus* originating from Argentina and Peru. The differences are so significant that the two populations can be said to belong to different sub-species. This study supports and extends previous work which has provided an increasing body of evidence that this nominal species is in reality a species complex. The use of the SIT has long been considered to be an option for the control of this species. Mass rearing and radiation sterilization protocols have now been developed, studies have been conducted on the sexual competitiveness of artificially reared flies and pre-release treatments have been developed in order to improve the sterile male competitiveness. The existence of considerable pre-zygotic isolation between different populations greatly complicates a regional approach to the use of sterile insects.

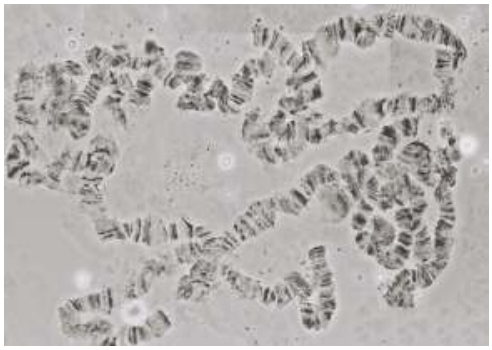
The data presented here for two *A. fraterculus* populations clearly show high levels of pre- and post-zygotic isolation, karyotypic and polytene chromosome anomalies and qualitative and quantitative differences in pheromone blends. Whilst each of these studies alone would be indicative of incipient speciation, taken together they provide a very strong case for a taxonomic revision of this species complex. The importance of this species as a major pest of fruit crops in many countries and its relevance as a quarantine pest make this revision essential and urgent.

3.3. Fruit Fly Genetics

In 2007 a series of detailed genetic and cytogenetic studies were carried out on genetic sexing strains in Bactrocera dorsalis *and* Bactrocera cucurbitae*. Both strains were developed in the USDA laboratory in Hawaii and were kindly made available to us by Dr. D. McInnis. They are presumably based on male linked translocations and use a white pupae mutant as a selectable marker. In the* ***2006 Activities Report****, data was presented on the mass rearing characteristics of the two strains and on their genetic stability. In 2007 the mitotic and polytene chromosomes were analysed in order to understand the chromosomal basis of the genetic sexing. Both strains do indeed carry male linked translocations but in* B. cucurbitae *the translocation is very complex resulting in a very low fertility of the strain. The low fertility would make the strain unsuitable for use in an SIT programme even though the strain is very stable.*

For several years the Unit has been supporting work being carried out at the Moscafruta facility in Tapachula, Mexico, where genetic sexing strains are under development for Anastrepha ludens*. In Tapachula, both a classical and a molecular approach are being used to achieve this goal. Important components of the classical approach include the isolation of visible mutations and the development of polytene chromosome maps. The group in Tapachula is now mapping several mutations including a dark pupae mutation that could have potential for the development of a genetic sexing strain. During 2007 the complete polytene chromosome map was produced in a collaborative effort and it will be published in 2008. A polytene chromosome map is a key tool for the development of genetic sexing strains using the classical approach.*

A mutation conferring temperature sensitivity has been vital in the development of classical medfly genetic sexing strain for operational SIT programmes. Although these mutations are very common following mutagenesis they are not easy to isolate or to work with but in Drosophila*, dominant temperature sensitive mutations have been isolated and cloned. These types of gene are highly conserved and when introduced into other species using transgenesis they should also be able to confer temperature sensitivity. In 2006 several transgenic medfly lines were produced which carried a dominant temperature sensitive mutation together with a fluorescent marker. Following several generations of characterization the strains were tested for temperature sensitivity producing mixed results. As heterozygotes none of the lines showed any lethality following a temperature shock. However as homozygotes various levels of lethality were observed but not to a level that would be useful in a genetic sexing strain.*



3.3.1. Cytogenetic Characterization of Genetic Sexing Strains in Two Fruit Fly Species[16]

Two strains, one for *Bactrocera dorsalis* and one for *Bactrocera. cucurbitae*, were isolated in Hawaii (Department of Entomology, University of Hawaii/USDA/ARS Pacific Basin Area Research Center) some years ago but very little detailed information is available on the strains. These strains were transferred to Seibersdorf for further analysis of quality control parameters **(Activities Report 2006)** and for genetic and cytological analyses. Both strains are based on Y-autosome translocations and the recessive autosomal *white pupae* (*wp*) mutation is used as a selectable marker. These strains have been maintained in the Unit for two years.

Polytene chromosomes have proven to be excellent material for studies related to the chromosome structure and function. Furthermore, analysis of polytene chromosomes allows the determination of the phylogenetic relationships of closely related species and can be used to distinguish members of sibling species. For insect pest species such studies can make a significant contribution both to the understanding of population variations and to the development/improvement of genetic pest control methods. The development of polytene chromosome maps for *Ceratitis capitata* has helped to improve the sterile insect technique (SIT) by aiding the analysis of translocation-based genetic sexing strains (GSS). Cytological analysis was performed using mitotic chromosomes from larval brain ganglia and larval salivary gland polytene chromosomes.

3.3.1.1. Cytology of the Bactrocera dorsalis genetic sexing strain

In one respect the autosomes of *B. dorsalis*, and the other *Bactrocera* species analysed so far, are distinctly different from those in medfly. The heterochromatin is much more concentrated in distinct parts of the chromosomes and not widely distributed as in medfly. This has significant consequences for the appearance of the polytene chromosomes. In *Bactrocera* salivary glands the heterochromatin aggregates and forms a loose chromocenter in which the autosomal centromeres are connected. This probably also contains the heterochromatic sex chromosomes because of their highly heterochromatic nature. This has also practical implications for the analysis of polytene chromosomes in GSS. In medfly the individual autosomes are usually found as separate entities and can be spread out more easily. However in *Bactrocera* species it is much easier to identify the centromeric region linked to the chromocenter. In medfly, *Bactrocera* species and probably also *Anastrepha* species the Y chromosome is not visible in polytene chromosomes isolated from salivary glands. However, in GSS it is very important that the Y chromosome be visible in order to determine the autosomal breakpoint. For the analysis of GSS in medfly polytene chromosomes had to be isolated from the two trichogen cells that produce the male-specific suborbital frontal bristles. In the trichogen cells the Y chromosome is visible as a heterochromatic block that is attached to the autosome at the site of the breakpoint. Currently it is not known whether a comparable situation exists in any other fruit fly species, i.e. whether the mapping of Y-autosome translocation breakpoints in species other than medfly is impossible.

During the analysis of the *B. dorsalis* GSS it was noted that the autosome involved in the translocation showed a very high degree of asynapsis. This phenomenon was not observed in medfly and may be used as an alternative method to determine the autosomal breakpoint without being able to visualise the Y chromosome. It is hypothesized that the existence of a chromocenter and/or the specific location of the autosomal breakpoint is the cause for the asynapsis. In *B.*

[16] E1.07, activity 7

dorsalis the translocated Y fragments will preferentially cluster in the chromocenter which then in turn also anchors the attached autosome fragments to this area. In contrast, the non-translocated autosome is linked to the chromocenter via its centromere.

Analysis of polytene chromosomes showed that only one autosome is involved in the translocation (**Figures 50 and 51**). The autosomal breakpoint is mapped very close to the centromere of this specific chromosome as it can be deduced from the pairing configuration of the two homologous chromosomes. The breakpoint is at the base of short arm and, therefore, this chromosome arm should be linked to the Y fragment carrying the centromere. Thus, although we cannot directly observe the Y chromosome in polytene nuclei we can map the translocation breakpoint on polytene chromosomes based on the hypothesis described above.

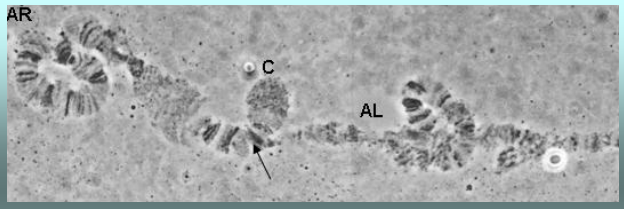


Figure 50. Polytene chromosome element involved in the translocation. Arrow indicates the autosome breakpoint on its right (AR) short arm. AL, represents the proximal part to the centromere (C) of the long arm of this chromosome.

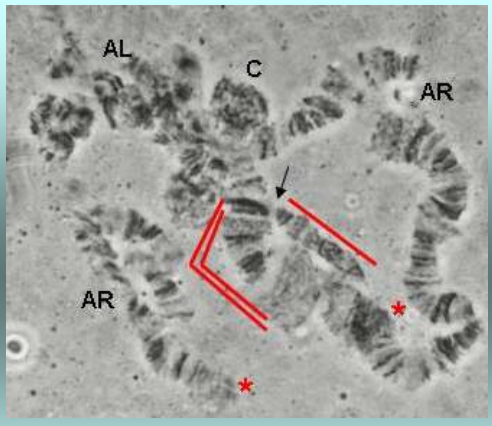


Figure 51. A triplication of the short arm, which starts from the breakpoint (arrow) to the tip of this arm. Note the extensive asynapsis of the long arm. C indicates the centromere of the chromosome.

In the *B. dorsalis* GSS *wp* is used as selectable marker and it can be expected that the autosome involved is homologous to chromosome 5 in medfly. Additional evidence for this conclusion is derived from a comparative analysis of the polytene chromosomes of the translocation strain with polytene chromosomes of *C. capitata.* This analysis showed a significant similarity in banding pattern of the translocated chromosome with the fifth chromosome of *C. capitata*; indicating that these chromosomes are indeed homologous.

The mitotic karyotype of a wild-type laboratory strain from Thailand is shown in **Figure 52** where sex chromosomes, X and Y, are indicated. The Y chromosome is very small as compared to the X chromosome. **Figure 53** shows a karyotype of the GSS strain where the X chromosome as well the Y-autosome translocation is indicated as T_1 and T_2. The intact autosome is shown as A. It is evident that the autosome involved in the translocation is a sub-metacentric one. The size and the pairing behaviour of the two fragments

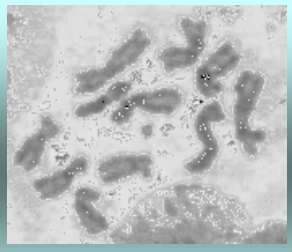


Figure 52. Mitotic karyotype of the wild type strain.

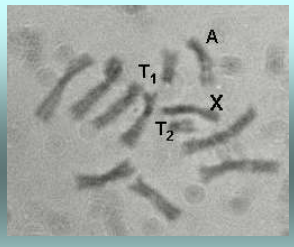


Figure 53. Mitotic karyotype of the GSS strain.

shown in **Figure 54** suggest that the autosomal breakpoint is close to the centromere but it is very difficult to say on which side.

During this analysis two exceptional karyotypes have been identified. The first one (**Figure 54**) has the small translocation fragment T_2, five pairs of autosomes and one X chromosome. That is, it has an autosomal triplication for the short arm of the autosome due to adjacent-1 segregation of the Y-autosome translocation during male meiosis. Adjacent-1 segregation leads to two types of genetically unbalanced offspring, one with a deletion of the region between the breakpoint and the tip of that arm and a second type where this region is triplicated. Offspring with a deletion usually die as embryos and reduce egg hatch. The triplication karyotypes are more viable and can survive even until the adult stage. The survival is dependent on the length of the triplication in combination with the sex of these individuals. The sex depends on the position of the breakpoint on the Y chromosome and on which chromosome, autosome or Y, contributes the centromere.

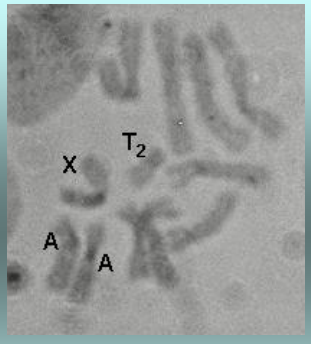


Figure 54. Mitotic karyotype of a triplication as it carries two intact autosomes (A) and the T_2 chromosome.

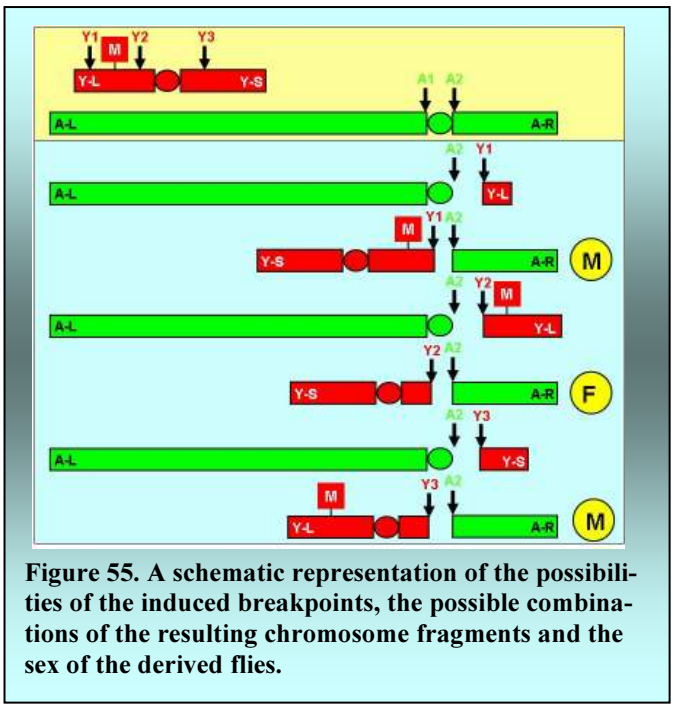


Figure 55. A schematic representation of the possibilities of the induced breakpoints, the possible combinations of the resulting chromosome fragments and the sex of the derived flies.

Figure 55 shows a schematic representation of the various possibilities. Two different autosomal breakpoints, both close to the centromere but in one case on the long (A1) and in the other on the short arm (A2), are considered in combination with the three principle breakpoint positions on the Y (Y1 to Y3). It is assumed that all combinations involving the autosomal breakpoint A1 are not viable: the translocation fragments with the autosomal centromere will result in deletions and will die as embryos while all fragments carrying the Y chromosomal centromere would be lethal because of the length of the resulting triplication. All combinations with the autosomal breakpoint A2 should lead to viable triplication type adjacent-1 offspring. The sex of these is determined by the Y breakpoint, i.e. is the breakpoint outside of the region between the Maleness factor and the centromere the resulting offspring would be male. Only if the breakpoint is between the Maleness factor and the centromere the resulting adjacent-1 offspring would be female. As indicated below the genetic data seem to suggest that the exceptional karyotype shown in **Figure 55** should be male.

The second exceptional karyotype (**Figure 56**) carries both translocation chromosomes, T_1 and T_2 and has in addition two X chromosomes. This could be the result of the non-disjunction of the two X chromosomes during female meiosis or a non disjunction of the sex chromosomes centromeres during male meiosis. Such individuals should be male and should be wild type with respect to *wp*.

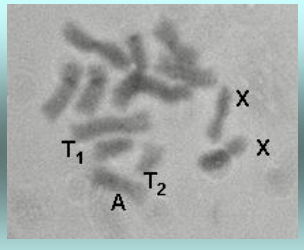


Figure 56.Mitotic karyotype with two X chromosomes.

3.3.1.2. Genetics of the Bactrocera dorsalis *genetic sexing strain*

The *B. dorsalis* GSS was reared under the same conditions as the medfly strains, i.e. the cage size was 19 x 20 x 32 cm and the medfly adult and larval diets were used. An egging bottle was used where the egging net was impregnated with Guava juice. The GSS was maintained for 18 generations under the standard rearing regime as it is also used for medfly strains; i.e. each generation 37 ml of pupae are used to set up the next generation without removing any aberrant flies while a parallel sample of 40 ml of pupae is screened. In 14 generations, 20 241 flies were screened, i.e. 1556 per 40 ml of pupae. In comparison, 40 ml of medfly pupae is equivalent to between 2200 and 2500 flies, depending on the strain used. The frequency of half emerged and crippled flies in the *B. dorsalis* strain is relatively high, i.e. 9.5% of the males and 13.8% of the females do not emerge completely or show some form of morphological abnormality. Among the 20 241 flies screened three recombinant females (wp^+) and nine recombinant males (*wp*) were detected. This represents an overall recombination frequency of 0.0445% (in VIENNA-8 the recombination frequency is 0.0143%). However, over the 18 generations analysed no accumulation of recombinants was observed. This may be due to the fact that this strain was reared under more relaxed and less selective conditions (i.e. 1440 flies/cage compared to VIENNA-8 with 2300 flies/cage).

In one generation the egg to adult viability was determined for 10 samples of 100 eggs collected either for 5h or 48h. The viability at different developmental stages was measured by counting egg hatch, the number of pupae and the number of adults (**Table 6**). In medfly the shorter egg collection produces a better recovery. However, this was not observed with the *B. dorsalis* strain. The data on the viability at the different developmental stages are in complete agreement with the result reported in the **Activities Report 2006**.

Table 6. Viability of *Bactrocera dorsalis* GSS

Egg collection 48 h						
Eggs	**Hatched Eggs**	***wp* Pupae**	**wp^+ Pupae**	**Females**	**Males**	**Overall Viabiliy**
1000	762	241	319	210	252	46.2%
Egg collection 5 h						
Eggs	**Hatched Eggs**	***wp* Pupae**	**wp^+ Pupae**	**Females**	**Males**	**Overall Viability**
1000	649	259	295	246	231	47.7%
Total						
Eggs	**Hatched Eggs**	***wp* Pupae**	**wp^+ Pupae**	**Females**	**Males**	**Overall Viability**
2000	706	250	307	228	242	47%

These data can also be used to deduce the structure of the translocation and to make some assumptions about its segregation behaviour. The overall egg to adult viability is 47% which indicates that a simple Y-autosome translocation involving only one autosome is present. Egg hatch is reduced to 71%. This reduction is caused by the embryonic lethality of the deletion type adjacent-1 karyotypes, i.e. a reduction in egg hatch by roughly 25% indicates that all four segregation types resulting from alternate and adjacent-1 segregation occur at equal frequency as is also the case in most medfly GSS. From the egg hatch data in combination with the data on adult recovery it also follows that the triplication type offspring survive the embryo stage. A large proportion of these karyotypes even reach the pupal stage which is indicated by the high number of brown pupae (i.e. more than the expected 250), the imbalance between white and brown pupae (250 *wp* versus 307 wp^+) and by the significant reduction of emergence of the males from brown pupae (*wp*: 91%; wp^+: 79%). The slight overabundance of males may indicate that a small proportion of the triplication types even reach the adult stage. However, that could be shown more directly if test crosses with a marker located on the long arm of the autosome could be done. In summary, adjacent-1 segregation produces males with a wp^+ phenotype which corroborates the results of the cytological analyses, i.e. in *B. dorsalis wp* is located on the short arm and the translocation breakpoint is also on this arm.

3.3.1.3. Cytology of the Bactrocera cucurbitae *genetic sexing strain*

Mitotic chromosome analysis was performed in both the GSS and a wild type lab strain from Bangladesh. The mitotic karyotype of the wild-type strain is shown in **Figure 57** where the X and Y chromosomes are indicated. The Y chromosome is smaller than the X chromosome. The mitotic karyotype of the translocation strain is shown in **Figure 58** in which the Y autosome translocation is indicated. The intact autosome is shown by "A" and the two translocation chromosomes by A-Y and Y-A. The X chromosome is also shown. The size of the two Translocated fragments, suggests that the autosome breakpoint is close to the centromere of a sub-metacentric chromosome. The small translocation chromosome has to carry most of the Y chromosome including its centromere while the second fragment carries the long arm of the autosome as well as the distal part of the Y chromosome.

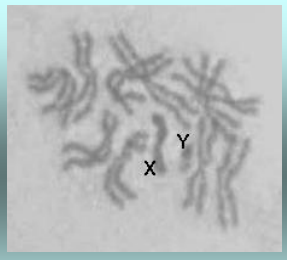


Figure 57. Mitotic karyotype of wild type strain.

Figure 58. Mitotic karyotype from the GSS strain.

As we have already mentioned above, the heterochromatin in salivary gland polytene nuclei of *Bactrocera* species aggregates and forms a loose chromocenter in which the autosome centromeres are connected. This structure probably contains the sex chromosomes as these are highly heterochromatic. This is also the case in polytene chromosomes of *B. cucurbitae*. This has consequences for the appearance of polytene chromosomes and helps the identification of autosomal centromeres. Furthermore, the banding pattern of *B. cucurbitae* polytene chromosomes is clearer as compared to the medfly polytene chromosomes and, therefore, the analysis is easier.

Polytene chromosome analysis in the GSS of *B. cucurbitae* revealed that the Y autosome translocation is more complicated than it was anticipated from the mitotic preparations. More specifically, in addition to the Y chromosome two autosomes are involved in the translocation

and not only one as suggested by the mitotic chromosome analysis. By analysing numerous polytene nuclei from both the GSS and the wild-type it is proposed that there are two breakpoints on the short arm of the first autosome, two on the long arm of the second autosome and at least one on the Y chromosome. Polytene chromosomes of the GSS are shown in **Figures 59** and **60**.

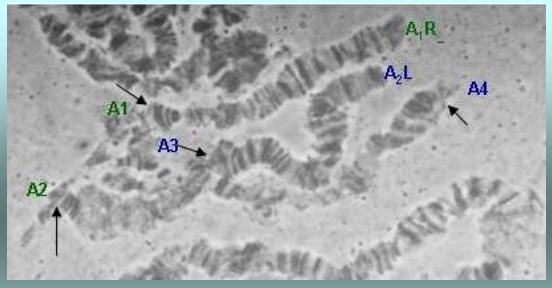


Figure 59. Polytene chromosomes from the GSS strain. Two autosomes are involved in the translocation, A_1 and A_2, with two breakpoints in each of them, A1 and A2 for the first one and A3 and A4 for the second.

Based on the characteristic pairing configurations of the homologous chromosomes in the heterozygous translocation the autosomal breakpoints were mapped. On some polytene chromosome preparations of the GSS strain a characteristic heterochromatic structure is connected to the translocation as shown in **Figure 60**. This structure shows similarities with the Y chromosome morphology observed in medfly trichogen polytene chromosomes. If this really represents the Y chromosome is the first time that a such structure is observed in salivary gland polytene chromosomes of the Tephritidae family, including the medfly where numerous Y-autosome translocations have been analysed**. Figure 61** shows the schematic representation of the breakpoints in the three chromosomes involved in the translocation and the most likely combination of the resulting chromosome fragments. These results are in full agreement with those from the quality control assessment (**Activities Report 2006**). The low overall viability (22%) in this strain is indicative for a Y-autosome translocation in which two autosomes are involved.

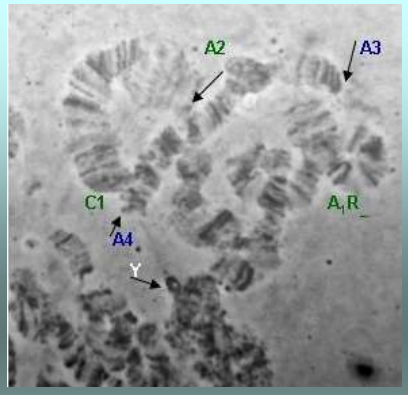


Figure 60. Polytene chromosomes from the GSS strain. Arrows indicate the breakpoints, A2, A3 and A4 of the autosomes. C1 shows the centromere of the first autosome. In the fragment A3-A4, a heterochromatic structure is connected which could be represent the Y chromosome.

In the *B. cucurbitae* GSS *wp* is used as selectable marker and it can be expected that the autosome involved is homologous to chromosome 5 in medfly. A comparative analysis of the polytene chromosomes of the translocation strain with polytene chromosomes of medfly showed a significant similarity in banding pattern of one of the translocated chromosomes **(Figure 61)** (A1) with the fifth chromosome of medfly indicating that these chromosomes are indeed

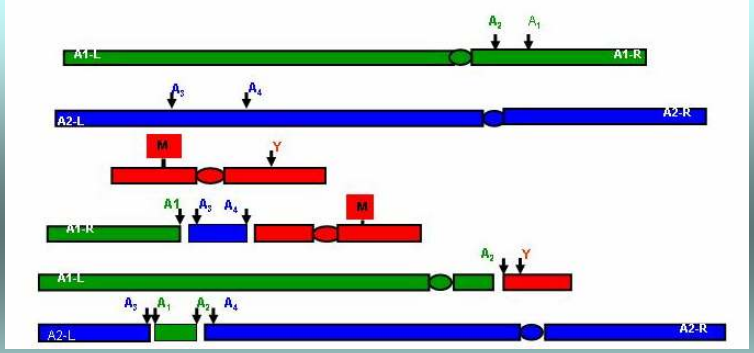


Figure 61. A schematic representation of the possibilities of the induced breakpoints and the combinations of the chromosome fragments to give the karyotype of the Y-autosome translocation strain.

homologous. It should be emphasized that the A1 chromosome shows also significant similarity with the chromosome involved in the translocation of the *B. dorsalis* GSS suggesting that these chromosomes are homologous to the chromosome 5 in medfly.

3.3.2. Analyses of Transgenic Medfly Lines [17]

The introduction of genetic sexing systems for medfly SIT has led to significant efficiency and performance improvements. There is a pressing, and well-recognised need for efficient sexing systems for SIT also for other species; for some, such as *Anopheles* mosquitoes, SIT may not be feasible without such a system. The medfly sexing system, based on classical radiation mutagenesis, is not easily transferable to other species. It would be desirable to establish a faster, more readily transferable approach, and transgenic methods may provide such a method.

One approach is to selectively kill females prior to release. For this purpose, some female-specific lethality is required, either female-specific expression of a lethal gene, or expression of a gene whose effect is female-specific. In any case, it is essential to be able selectively utilise the system, to allow production of males and females in the initial rearing generations, to amplify the strain, then to remove females from the final release generation. This requires that the transgenic system can be specifically induced, or de-repressed in the final generation. In Drosophila, missense mutations in the genes encoding the β2 and β6 subunits of the 20S proteasome subunit, lead to the production of dominant temperature-sensitive (DTS) "poison subunits" or antimorphic mutations, among them DTS5 and DTS7. In such mutant flies, raised at the restrictive temperature of 29ºC, embryonic and larval development appears to proceed normally, but metamorphosis is abnormal (failure of the adult abdominal segmentation, head evasion disrupted etc.) and death occurs at the late pupal stage.

3.3.2.1. Transformation with different piggyBac *vectors*

In collaboration with A. Handler (USDA, Gainesville, USA) experiments were conducted to study the potential of these dominant lethal genes to develop strains for the population suppression of tephritid fruit fly pests in SIT programmes. Constructs carrying the DTS5, DTS7 and derivatives thereof were generated and used to genetically transform the wild-type strain EgII of the medfly. In the vectors used, DTS5 and DTS7 both isolated from *Drosophila*, were either under control of their own (*Drosophila*) or a medfly heatshock promoter. In addition these constructs carry either EGFP or DsRed as a marker to be able to identify the respective transformants. In all experiments *piggyBac* was used as the transformation vector. The constructs were micro-injected into medfly embryos together with an appropriate helper plasmid that provided the transposase activity in *trans*. **Table 7** shows a summary of the injection experiment.

Table 7. Statistics of three micro-injection experiments

Vector	Description	Eggs Injected	Embryos	Pupae	Adults	% Overall Recovery
#249	**pBac/hspDTS7/EGFP**	864	452	323	291	33.7
#348	**pBac/hspDTS5/DsRed**	650	118	60	58	8.9
#350	**pBac/DTS5/DsRed**	720	443	345	311	43.2

[17] E1.06, activity 7

In two cases the overall recovery was very good. As a comparison, **Figure 62** shows the summary of all injection experiments that were performed so far. Using either the *w wp* or the EgII strain an overall survival of ca 23% can be obtained. Injecting D53 produces a much lower recovery (3%) which is probably due to the fact that this strain is temperature sensitive and the heatshock applied during the transformation seems to have a negative effect.

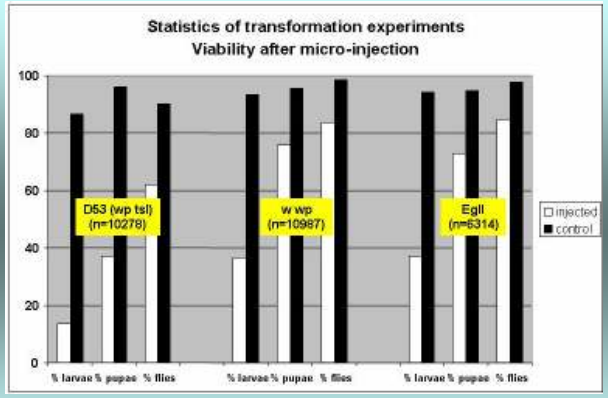


Figure 62. Summary of all injection experiments using 3 different strains. Survival is expressed as percent of the previous developmental stage.

All surviving G_0 flies were crossed with wild type (EgII) or with *w wp* flies. All eggs of these crosses were collected and all resulting G_1 flies were screened. In **Table 8** the cages are listed that produced fluorescing individuals; i.e. 46, 12 and 24 potential transgenics were recovered, respectively. In brackets the number of cages is shown that did not produce any transgenic offspring.

Table 8. Recovery of fluorescent G1 individuals

#249 (4 cages)		#348 (1 cage)		#350 (9 cages)	
G_0 flies / cage	Fluoresc. G_1	G_0 flies / cage	Fluoresc. G_1	G_0 flies / cage	Fluoresc. G_1
4	2	36	12	4	2
25	15			25	2
25	1			26	9
25	3			23	5
26	3			50	4
27	6			44	2
41	3				
45	7				
43	4				
15	2				
276	46	36	12	172	24

Fluorescing G_1 flies were mated individually. For at least three to four generations the transgenics lines were maintained as heterozygotes, i.e. each generation fluorescent flies were crossed with flies from the wild type strain EgII or *w wp*. Eventually after the first temperature tests, the transgenes were made homozygous. This was done by inbreeding fluorescent flies. In the next generation it was attempted to separate homozygous from heterozygous flies based on the intensity of the fluorescence. However, that was not always successful especially in cases where the fluorescence is very weak (see below). Such strains were inbred and selected for more than seven generations; i.e. each generation flies with the strongest fluorescence (presumed to be the homozygotes) were selected for inbreeding. Like in all other injection experiments we can distinguish four different patterns of fluorescence (A to D in **Figure 63**).

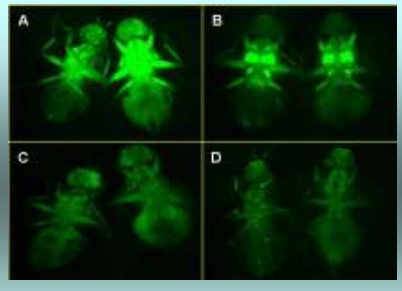


Figure 63. Different EGFP expression, independent of the vector used.

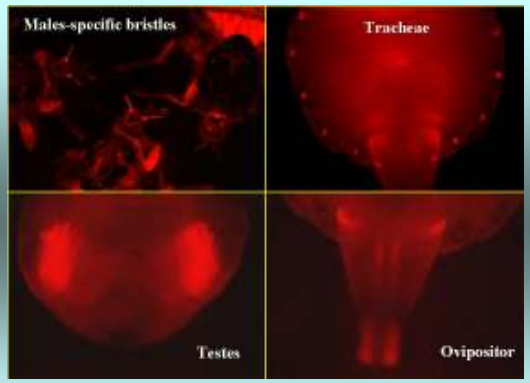


Figure 64. Exceptional, tissue-specific fluorescence patterns.

Within each class the intensity of the fluorescence varies from strain to strain. This is indicated by superscript numbers where 5+ means very strong and 1+ means very weak. On occasion strains are found where the fluorescence is restricted to particular cells/tissues (**Figure 64**). Interestingly, two of these exceptional patterns (ovipositor and testes) were detected among the families obtained in the injection experiment with vector #348 described below.

3.3.2.2. Transformation with the vector #348 (pBac/hspDTS5/DsRed)

All lines were tested genetically to determine whether the insertion is located on the 5th chromosome. However, none showed link to this autosome. All lines were classified into the four patterns shown in **Figure 64**. Furthermore, most of the strains were analysed via Southern hybridization to determine primarily how many insertions are present. **Table 9** shows that only 7 strains represent independent transformation events and that most of the strains have two or even three insertions.

Table 9. Analysis of transgenic lines injected with vector #348

Strain	Pattern/Intensity	Southern Results	Identical to
4-1M 1m	C^{4+}	2 ins = 12kb and 6kb	
4-1M 2m	C^{2+}	3 ins = 12kb, 9.5kb, 6.5kb	= 3m/7m
4-1M 3m	C^{2+}	3 ins = 12kb, 9.5kb, 6.5kb	= 2m/7m
4-1M 4m	B, ovipositor	1 ins = 6.2 kb	= 3f
4-1M 5m	B		
4-1M 6m	B, testes	2 ins = 12kb, 8.8kb	= 1f
4-1M 7m	C^{5+}	3 ins = 12kb, 9.5kb, 6.5kb	= 2m/3m,
4-1M 8m	C^{2+}	1 ins = 10kb	
4-1M 1f	B, testes	2 ins = 12kb, 8.8kb	= 6m
4-1M 2f	B^{3+}, legs		
4-1M 3f	B, ovipositor	1 ins = 6.2 kb	= 4m

Table 10. Temperature tests with heterozygous lines transformed with pBac/hspDTS5/DsRed

Strain	No. Eggs	No. Larvae	No. Pupae	Adult Fluorescence	
				+ve	-ve
4-1M 1m	200	167	160	69	72
4-1M 2m	200	159	152	54	65
4-1M 3m	200	175	154	59	78
4-1M 4m	200	181	172	68	80
4-1M 5m	200	162	132	49	59
4-1M 6m	200	164	143	64	69
4-1M 7m	200	188	155	70	73
4-1M 8m	200	178	160	75	73
4-1M 1f	200	187	172	84	81
4-1M 2f	200	168	144	64	62
4-1M 3f	200	188	111	67	19
EgII	200	174	149		130

The first temperature tests with 11 of the available 12 lines were done in G_3 of the outcrossing with EgII, i.e. the transgenes were heterozygous (**Table 10**). Eggs were collected for 5h and put on standard larval diet in Petri dishes. Until 3rd instar the rearing temperature was 25°C. At this stage a 1h heat shock with 39°C

was applied. The rest of the rearing was done at 30°C. **Table 11** shows that none of the heterozygous strains showed any temperature dependent lethality of the transgenic flies, i.e. none of the families shows a significant reduction of the fluorescent flies as compared to the non-fluorescent ones.

Six of the twelve lines were made homozygous. However, due to the fact that most of them carry several insertions this is not completely certain. With these six lines two different temperature tests were conducted and compared to the control at 25°C. Larvae were reared at 25°C until 3rd instar. Then the rearing temperature was increased to 30°C either with or without a 1h heat shock at 39°C (**Table 11**). It is obvious that in five of the six strains the increased rearing temperature reduces the adult recovery. In the most extreme case (4-1M 6m) the lethality occurs at the developmental stage expected for the DTS5 mutation, i.e. adult emergence is reduced significantly while the recovery of pupae from hatched eggs is normal (87 to 97%). However, even this significant lethality would not be sufficient for a practical application. This type of temperature sensitive lethality will probably not be suitable for genetic sexing in medfly.

Table 11. Temperature tests with homozygous lines carrying pBac/hspDTS5/DsRed

	25°C		25°C/30°C			25°C/39°C/30°C		
Strain	**Eggs**	**Adults**	**Eggs**	**Adults**	**% Recovery (relative 25°C)**	**Eggs**	**Adults**	**% Recovery (relative 25°C)**
4-1M 1m	100	64	100	35	54.7	430	79	28.7
4-1M 2m	100	32	100	10	31.3	403	22	17.1
4-1M 3m	100	64	100	37	57.8	406	28	10.8
4-1M 4m	100	53	100	50	94.3	408	116	53.6
4-1M 5m	100	24	100	22	91.7	387	111	119.5
4-1M 6m	100	82	100	11	13.4	431	18	5.1

3.4. Mosquito Rearing and Genetic Sexing

Several significant developments in all areas; from the laboratory, in development of field sites and personnel changes occurred in 2007. The project is rapidly moving toward the point that technologies and information must be implemented at field sites thus focusing the activities on specific goals. Toward that end, significant advances have been made in sexual sterilization and its effect on competitiveness, mass rearing for adults, and conventional genetic sex separation.

The isolation of a genetic sexing strain in Anopheles arabiensis has been a major breakthrough in 2007. Mosquito SIT cannot be implemented without an efficient mechanism to remove the sterile females before the males are released. In many mosquito species these strains have been developed mostly using insecticide resistance genes and male linked translocations. In these systems an insecticide resistance gene is linked to the male sex using a radiation induced translocation. A similar approach was taken in An. arabienis using dieldrin resistance and a genetic sexing strain was isolated. The strain is quite stable and has been reared for many generations without the need to remove recombinant individuals. The stability is probably due to the presence of complex translocation which reduces the fertility to about 25%. This is a fairly serious problem in terms of efficient mass rearing, nevertheless sufficient males could be reared so that a small scale release of sterile male mosquitoes could be carried out in Sudan.

The use of stable isotopes is providing an opportunity to improve the monitoring of sterile insects in the field and now also to identify whether a wild female has mated with a released male. This was shown to be possible by analyzing isotope ratios in spermathecae of mated and virgin females, the females had either mated with unlabelled males or those labeled with ^{15}N. The relatively high cost of the analysis would limit this approach to selected evaluations of field collected females. It would however provide very important information for programme evaluation.

Many activities were carrier out in Sudan in 2007 which required considerable input from Unit staff both in terms of expert visits and provision of material and advice. The genetic sexing strain was successfully transferred to the counterpart and the semi-field cage system made operational. In addition the adult mass rearing cage was constructed in Seibersdorf and shipped to Khartoum. Excellent baseline data on larval breeding sites in the project area has been collected for two years and this information will be used to make assessments of the size of the mosquito population. In 2008 preliminary plans will be made for the construction of a large rearing facility and the information on the size of the target population is essential for the design of the facility.

3.4.1. Stable Isotope Labeling of Semen for Mating Studies [18]

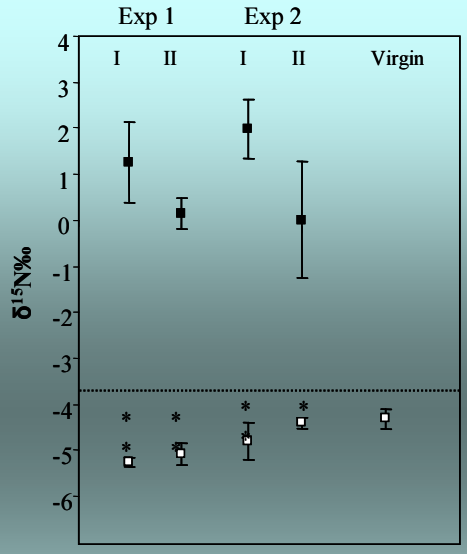


Figure 65. Labelling semen in the spermathecae of females.

Mean ± s.e.m. δ15N‰ values of inseminated (filled) and uninseminated (open) spermathecae for two experiments. Dotted line indicates the threshold value of 3 s.d. above mean δ15N‰ of virgin females. Virgin samples are included. Dissection treatments are: I, females dissected immediately after mating; II, females isolated and dissected 3 days after mating. Asterisks indicate significant difference between positive and negative spermathecae for each treatment at * P< 0.05, and ** P< 0.01.

In the previous annual report we discussed the successful application of the use of the stable isotope ^{13}C to study mosquito mating by tracing the fate of labeled semen into spermathecae. We also reported that initial experiments using ^{15}N as a label did not result in sufficient detection of the label in spermathecae. These experiments were extended during 2007 and positive results were obtained. Briefly, ^{15}N-labelled glycine was added to the larval diet, and the transfer of ^{15}N labeled semen determined by mass spectrometry. After mating, spermathecae were analyzed for isotope ratios. Results demonstrated that spermathecae positive for semen could be distinguished from empty ones or controls (i.e. filled with unlabelled semen) using the raw δ ^{15}N values **(Figure 65)**. Hence ^{15}N-labelling is feasible and, in combination with ^{13}C, can be used to study competitiveness and multiple mating events.

3.4.2. Comparing the Competitiveness of Males Irradiated as Pupae or Adults [19]

Competitiveness of males irradiated in the pupal or adult stage was determined in small and large cages. Two doses were used, a semi-sterilizing dose of 70 Gy and a fully sterilizing dose of 120 Gy. Results showed that overall, adult irradiation resulted in greater competitiveness compared to pupal irradiation; and males were equally competitive to unirradiated males at low doses when tested in the large cage. Males irradiated with the higher dose had a lower competitiveness compared to unirradiated males when tested in the smaller cage.

Males irradiated with the lower dose in the pupal stage had a better competitiveness when experiments were performed in small cages compared to the larger cages. Males irradiated with the higher dose were significantly less competitive compared to unirradiated males in both cages. The fact that cage size affects competitiveness means that these studies must be extended to semi-field and field releases.

3.4.3. Sperm Morphology and Quantity [20]

Previous studies have demonstrated differences in the sperm length of mosquitoes of different genera and changes in those stored in the spermathecae. Studies to determine the degree of sperm

[18] F1.04, activity 10
[19] F1.04, activity 1
[20] F1.04, activity 3

length polymorphism were undertaken in the Dongola strain of *An. arabiensis*, and results were compared to the distribution observed in wild mosquitoes collected from the field. Sperm length polymorphisms were present in *An. arabiensis,* and sperm tail length varied between < 50-500 µm. Males from the laboratory strain had a similar distribution compared to wild males and distributions were similar compared to the closely related sibling species *An. gambiae*. Sperm quantity was determined based on a protocol used in *Aedes aegypti*. Results showed that the quantity of sperm in the testes increased with male age.

3.4.4. A Classical Genetic Sexing System Based on a Conditionally Lethal Allele [21]

A genetic sex-separation strain for *Anopheles arabiensis* has been produced based on a classical approach. The conditionally lethal allele confers resistance to the insecticide dieldrin; a cyclodiene and one of the early organochlorine insecticides developed in the 1940s. Because dieldrin is very stable, it a persistent pollutant that will bioaccumulate; therefore it has been banned since the 1970s. However, as there is no prospect of dieldrin being used for control purposes in the future, the mass production and release of sterile males resistant to dieldrin does not present a major concern for its use in a controlled laboratory or production environment or its use for sex separation.

A stock named GSS 5-33 was created during 2007. This stock allows almost complete removal of females by exposure of larvae to dieldrin. The rate of recombination between the translocation and dieldrin resistance has been determined to be < 0.4%. Recombination usually results in the dissociation of the dieldrin resistance allele from the male-determining Y chromosome. However, this stock was maintained without selection for the proper arrangement without losing it for 9 generations. While it is too early to reach a firm conclusion, it is possible that the stock is highly stable in spite of the observed recombination or that the recombination frequency is lower than 0.4%. Studies are under way to analyze the genotype of the rare females. While this strain is highly valuable, the chromosome rearrangements resulted in 74% sterility and we will attempt to create additional stocks to consider as alternatives.

3.4.5. Detecting the Dieldrin Resistance Marker in Sperm of Mated Females [22]

A PCR assay has been developed which is capable of detecting the single nucleotide change responsible for resistance to dieldrin (*Rdl*). This enables identification of released males, their sperm in females, and resistant individuals. A simple PCR to distinguish the different resistance phenotypes in *An. arabiensis* has already been developed. This was used as the starting point to develop simplified assays, first for the identification of dieldrin resistant males based on a tarsus, and secondly to determine the *Rdl* genotype in sperm from spermathecae. Chitinase treatment of dissected spermathecae at 37ºC followed by lysis buffer effectively released the sperm. The method is simple, rapid and amenable to high throughput with scope to be further developed for the use in the identification of sterile males in the field on larger scale.

[21] F1.04, activity 11
[22] F1.04, activity 10

3.4.6. Georgia Tech Collaboration [23]

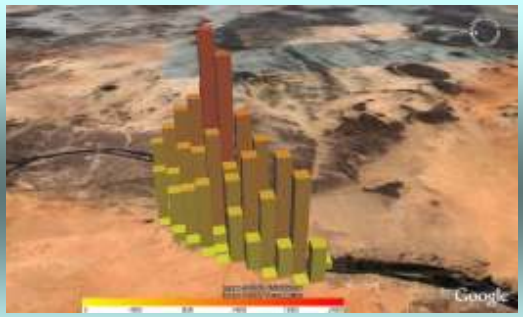


Figure 66. Predicted mosquito population levels.

Before returning to the IAEA, Mr. Mark Benedict initiated collaboration with Georgia Institute of Technology Industrial and Systems Engineering seniors and faculty to develop an overall plan for the mosquito SIT project in Sudan. This project was conducted as a required capstone achievement of the student's bachelor's degree. The project emphasized optimization, logistics, population size estimation, and consideration of different locations for the virtual production facility. Out of 21 projects performed during the semester, the Mosquito SIT project was selected as the winner in the departmental competition. One part of the project was a 'sales package' for potential donors. **Figure 66** shows the relative population levels predicted along a target region of the River Nile. This information will be used to help identify the size of the first release zone and also to help plan the design of a rearing facility.

3.4.7. Sudan Activities [24]

The Sudan team has been extremely active and is gathering excellent pre-release data that is now sufficient to begin increasing the scale of releases and determining a specific release strategy. During two weeks of March/April, experiments in Sudan were undertaken to 1) demonstrate the irradiation process and transport adult mosquitoes to the field site by air; 2) prepare the field cage in Dongola for experiments and test the survival and mating of mosquitoes in a field cage. The field cage (**Figure 67**) was equipped with resting sites and vegetation to create favorable places for mosquitoes where humidity was higher and temperature lower compared to ambient conditions. Results demonstrated that the small-scale irradiation at 70 Gy and transportation of insects to the field site is feasible. Field cage experiments showed that insects survived and mated in the cage, and studies on the competitiveness of irradiated males should be pursued.

Figure 67. Field cage in Sudan.

These field cage releases were paralleled by releases conducted by the Sudan team **(Figure 68)** which constituted the first release of sterile *A. arabiensis* males in the Northern State. A small release of approximately 1600 sterile males was made in November. Surveillance activities conducted during the following two days recovered five males, two of which were marked released males. The release was attended

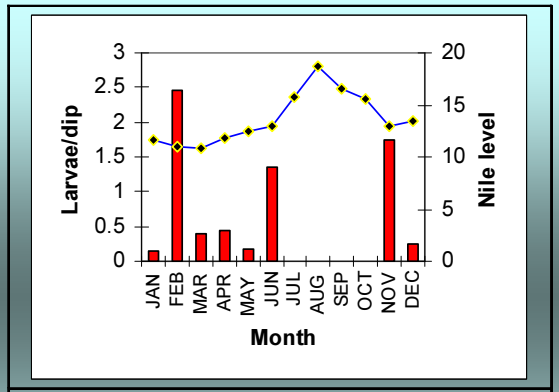
Figure 68. Sudanese field release team.

[23] unplanned activity
[24] F1.04, activity 13

by local leaders and representatives from the Malaria Administration. This release further confirmed the transportability of adults from Khartoum to the field site with little or no mortality.

In order to give a clear view about the spatial and temporal pattern of *A. arabiensis* breeding sites in Northern Sudan, Geographical Information Systems (GIS), Global Positioning Systems (GPS) and Remote Sensing (RS) were used in an intensive study between May 2005 and May 2007. A cross sectional longitudinal larval survey study was carried out in two localities (Dongola and Merowe), each in typical anticipated release areas in Northern State of Sudan.

A. arabiensis immature stages were collected all year but with seasonal fluctuations. The highest densities of larvae were recorded during December to April in Dongola and December to February in Merowe areas while the lowest densities were recorded between May and October in both areas. During the low stage of the Nile River the number of positive breeding site and larval density were high, while during flood periods larvae were collected only from manmade sites which had low density. This strong and predictable seasonal fluctuation in population sizes is expected to be useful as a natural means to take advantage of SIT's efficiency in low populations. Thus September – October will be the most suitable period to begin releases in Northern Sudan.



Merowe natural breeding sites by month (red bars) and the Nile River level gauge height (blue line). Extremely low numbers of mosquitoes were observed during the high stages of the River, though this effect was not as extreme in Dongola.

Figure 69. Larval density and River Nile level.

An inverse relationship was found between *A. arabiensis* larvae occurrence in site and River Nile levels distance of breeding sites from the river and distance of breeding site from the nearest settlements in the other site **(Figure 69)**. The highest percentages of positive breeding sites were found within 200 m from settlements and 1 Km from the River Nile.

4. TRAINING ACTIVITIES

Two fellows, one from Ethiopia and one from Tanzania have been trained on molecular biology techniques related to the tsetse virus. The first fellow from Ethiopia was trained on the virus purification by sucrose gradient, protein analysis by PAGE, DNA extraction from legs and flies, PCR and quantitative PCR. The second fellow from Tanzania was trained on the virus purification and DNA extraction. Three fellows from Kenya were trained on irradiation and dosimetry in relation to tsetse SIT. Training has continued on the TPU3 tsetse rearing system for fellows from Ethiopia and Tanzania.

A fellow from Egypt was trained on the characterization of the production and QC profile of two genetically modified medfly strains. The standard genetic sexing strain, based on the temperature sensitive lethal mutation and a translocation, was used as reference. The strains included in this study were V-8/2 and V-8/4. Both strains are normal temperature sensitive genetic sexing strains that carry in addition a DsRed marker gene which was introduced by means of genetic transformation. In addition, the fellow was trained on cytological techniques. Polytene and mitotic chromosome preparations were made from *Ceratitis capitata*, *Bactrocera cucurbitae* and *Bactrocera dorsalis.*

5. APPENDICES

5.1. Staff Publications

ABD-ALLA, A, H BOSSIN, F COUSSERANS, **A PARKER,** M BERGOIN, **A ROBINSON,** Development of a non-destructive PCR method for detection of the salivary gland hypertrophy virus (SGHV) in tsetse flies, J. Virol. Methods **139** (2007) 143-9.

ABD-ALLA, AMM, F COUSSERANS, **AG PARKER,** JA JEHLE, NJ PARKER, J M VLAK, **AS ROBINSON,** M BERGOIN, Genome analysis of a *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV) reveals a novel large double-stranded circular DNA virus, J. Virol. (in press).

BENEDICT, MQ, AS ROBINSON, "Impact of technological improvements on traditional control strategies", Transgenesis and Management of Vector-Borne Diseases (S AKSOY, Ed.), (2008) (in press).

BRICEÑO, RD, WG EBERHARD, **AS ROBINSON,** Copulation behaviour of *Glossina pallidipes* (Diptera: Muscidae) outside and inside the female, with a discussion of genitalic evolution, Bull. Entomol. Res. **97** 5 (2007) 471-88.

CACERES, C, DO MCINNIS, TE SHELLY, EB JANG, **AS ROBINSON,** J HENDRICHS, Quality management systems for fruit fly (Diptera: Tephritidae) sterile insect technique, Fla. Entomol. **90** 1 (2007) 1-9.

CACERES, C, E RAMIREZ, **V WORNOAYPORN, SM ISLAM, S AHMAD**, A protocol for storage and long-distance shipment of Mediterranean fruit fly (Diptera: Tephritidae) eggs. I. Effect of temperature, embryo age, and storage time on survival and quality, Fla. Entomol. **90** 1 (2007) 103-9.

CHANG, CL, **C CACERES,** S EKESI, Life history parameters of *Ceratitis capitata* (Diptera: Tephritidae) reared on liquid diets, Ann. Entomol. Soc. Am. **100** 6 (2007) 900-6.

CORONADO GONZALEZ, PA, S VIJAYSEGARAN, **AS ROBINSON,** Mouthpart structure and feeding mechanisms of adult *Ceratitis capitata* (Wied) (Diptera: Tephritidae), Insect Science (2008) (in press).

GARIOU-PAPALEXIOU, A, G YANNOPOULOS, **AS ROBINSON, A ZACHAROPOULOU**, Polytene chromosome maps in four species of tsetse flies *Glossina austeni*, *G. pallidipes*, *G. morsitans morsitans* and *G. m. submorsitans* (Diptera: Glossinidae): a comparative analysis, Genetica **129** 3 (2007) 243-51.

HELINSKI, ME, R HOOD-NOWOTNY, L MAYR, BG KNOLS, Stable isotope-mass spectrometric determination of semen transfer in malaria mosquitoes, J. Exp. Biol. **210** 7 (2007) 1266-74.

HELINSKI, ME, BG KNOLS, Mating competitiveness of male *Anopheles arabiensis* mosquitoes irradiated with a partially- or fully-sterilizing dose in small and large laboratory cages. J. Med. Entomol. (in press).

HENDRICHS, J, P KENMORE, **AS ROBINSON,** MJB VREYSEN, "Area-Wide Integrated Pest Management (AW - IPM): Principles, Practice and Prospects", Area-Wide Control of Insect Pests. From Research to Field Implementation (MJB VREYSEN, AS ROBINSON, J HENDRICHS, Eds.), Springer, Dordrecht, The Netherlands (2007) 3-33.

HOOD-NOWOTNY, R, BGJ KNOLS, Stable isotope methods in biological and ecological studies of arthropods, Entomol. Exp. Appl. **124** 1 (2007) 3-16.

KNOLS, BG, HC BOSSIN, WR MUKABANA, **AS ROBINSON,** Transgenic mosquitoes and the fight against malaria: managing technology push in a turbulent GMO world, Am. J. Trop. Med. Hyg. **77** 6 Suppl (2007) 232-42.

M'SAAD GUERFALI, M, A RAIES, H BEN SALAH, F LOUSSAIEF, **C CACERES,** "Pilot Mediterranean fruit fly *Ceratitis capitata* rearing facility in Tunisia: Constraints and prospects", Area-Wide Control of Insect Pests. From Research to Field Implementation (M J B VREYSEN, AS ROBINSON, J HENDRICHS, Eds.), Springer, Dordrecht, The Netherlands (2007) 535-543.

MALCOLM, CA, DA WELSBY, BB EL SAYED, "SIT for the malaria vector *Anopheles arabiensis* in Northern State, Sudan: an historical review of the field site", Area-Wide Control of Insect Pests. From Research to Field Implementation (MJB VREYSEN, AS ROBINSON, J HENDRICHS, Eds.), Springer, Dordrecht, The Netherlands (2007) 361-372.

MAMAN, E, **C CACERES,** A protocol for storage and long-distance shipment of Mediterranean fruit fly (Diptera: Tephritidae) eggs. II. Assessment of the optimal temperature and substrate for male-only production, Fla. Entomol. **90** 1 (2007) 110-4.

PARKER, AG, K MEHTA, Sterile insect technique: a model for dose optimization for improved sterile insect quality, Fla. Entomol. **90** 1 (2007) 88-95.

PARKER, NJ, **AG PARKER,** Simple tools for assembling and searching high-density picolitre pyrophosphate sequence data, Source Code Biol. Med. (2008) (in press).

WILKINS, EE, SC SMITH, JM ROBERTS, **M BENEDICT,** Rubidium marking of *Anopheles* mosquitoes detectable by field-capable X-ray spectrometry, Med. Vet. Entomol. **21** 2 (2007) 196-203.

5.2. Staff Travel

Staff Member	Destination	Period	Purpose of Travel
Abd Alla, Adly	Neustad, Germany	20 Apr	Discussion on virus sequence.
	Quebec City, Canada	12-16 Aug	To participate in the 40th Annual Meeting of the Society of Invertebrate Pathology and 1st International Forum on Entomopathogenic Nematodes and Symbiotic Bacteria, Université Laval-Quebec City, Canada.
Boigner, Rudolf	Bratislava, Slovakia	31 May	To observe blood collection procedure.
Caceres, Carlos	Valencia, Spain	5-9 March	To give technical assistance to the "Empresa de Transformasion Agraria" (TRAGSA) on Medfly mass rearing protocols and quality control activities of the Vienna-8 genetic sexing strain.
	Fresno, CA, USA	24-27 April	To attend and present the Entomology Unit's results at the 9th Exotic Fruit Fly Symposium. Fresno, Ca.; and to visit USDA, ARS, SJVASC, Parlier, Ca.
	Juazeiro, Brazil	8-13 Jan	To advise Brazilian Medly Programme on the mass rearing of VIENNA 8 GSS.
Helinski, Michele	Khartoum, Sudan	24 March-7 Apr	Establish rearing system in the facility in Khartoum.
	Khartoum, Sudan	3 Nov-7 Nov	Evaluation of methods in use for semi-field cage studies on competitiveness of sterile males and advise and assist with development of methods for scaling up of insect irradiation in preparation for field release.
	Manaus, Brazil	3-16 June	To attend "Biology of Disease Vectors" course.
Kabore, Idrissa	Luanda, Angola	1-5 Oct	Attend and make a presentation at the ISCTRC meeting.
Malcolm, Colin	Manchester, UK	28 Feb-2 March	Discuss potential potential collaboration on transgenic mosquitoes.
	Havana, Cuba	8-11 Aug	Attend Dengue and Aedes Symposium.
	New Haven, CT, USA	13-14 Aug	Discuss collaboration and present a talk.
	Reunion, France	3-5 Dec	Attend symposium on chikungunya and dengue.
	Khartoum, Sudan	18-28 March	Project evaluation and assist in the small scale field release of sterile mosquitoes.
Mohammed, Hasim	Bratislava, Slovakia	25 Jan	Observe on blood collection and advise on handling procedures.
	Bratislava, Slovakia	31 May	Observe on blood collection and advise on handling procedures.

Parker, Andrew	Montreal, Canada	28 Oct-2 Nov	To organize a workshop on "Sterile Insect Production and Quality Control" and to moderate the session "Production of sterile insects and their quality control" in an IOBC-AMRQC meeting.
	Orlando, FL, USA	5-6 Nov	Discuss aerial release machine for tsetse with engineering company.
	Montpellier, France	2-4 April	To consult with Dr Max Bergoin on the tsetse salivary gland hypertrophy virus, and to attend the Leverhulme Trust Tsetse Research Network meeting.
	Nairobi, Kenya	7-11 May	To attend, as Scientific Secretary, the 3rd RCM for the CRP D4.20.10.
	Raleigh, NC, USA	29 Jan- 2 Feb	Lecture on dosimetry to a course on "Irradiation as a phytosanitary treatment".
	Atlanta, GA, USA	5 Feb	Discussions with the RadSource Co. the development of an x-irradiator system for bacterial decontamination of tsetse blood diet.
	Charlottesville, VA. USA	6 Feb	Discussions on the tsetse release machine.
	Kaliti, Ethiopia	26 Feb-2 March	Discussions on the installation of module 2 and problem solving for TPU 3.
	Kaliti, Ethiopia	19-23 Nov	Discussions on the installation of module 2 and problem solving for TPU 3.
Robinson, Alan	Liverpool, UK	21-23 June	To attend an EU tsetse planning meeting in relation to mating compatibility studies on Glossina fuscipes, To attend a Gates consortium meeting on novel odour baits for *Glossina palpalis*.
	Geneva, Switzerland	23-25 April	To participate in a WHO/TDR panel to develop a strategic research programme for vector control. Attend PAAT/ PATTEC coordination meeting.
	Daegu, South Korea	19-24 Aug	To attend, as an invited speaker, the ICIBI (International Congress of Insect Biotechnology & Industry) meeting.
Soliban, Sharon	Wroclav, Poland	2-4 July	To attend the "INWES Regional Symposium on Women Scientists and Engineers in New EU Countries and Eastern Europe - Strategies for a highly skilled global workforce".
	Gent, Belgium	29 Sept-7 Oct	To test live and formulated feeds and rearing set-ups used in artemia culture on mosquito larvae for future use in Seibersdorf.
	Atlanta, GA, USA	12-16 Feb	Training on advanced rearing techniques, introduction to molecular based authentication techniques for *Anopheles arabiensis* strains relevant for the mosquito SIT project.

5.3. 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

Institution	Topic
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
Oxitec Ltd, 71 Milton Park, Oxford OX14 4RX, UK	Transgenic strain evaluation
Moscafruta Program, 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
Zoologisches Institute, University of Vienna, Althaustrasse 14, A-1090 Vienna, Austria	Tsetse sound production

5.4. Trainees, Fellows and Scientific Visitors

Name	Project	Months/Days	From	To
Fellows				
MUSIE, Mr K	ETH/06004	3/04	2006-04-05	2007-04-04
ARNOLDS, Mr LE	SAF/06013	0/22	2006-07-03	2007-01-22
KYELA, Mr MS	URT/06026	2/24	2006-09-25	2007-03-24
OSAE, Mr M	GHA/06011	3/15	2006-10-15	2007-04-15
JEMUTAI, Ms M	KEN/06028	0/05	2007-01-02	2007-01-05
KAHIGA, Mr DT	KEN/06026	0/05	2007-01-02	2007-01-05
KHIDER, Ms HBB	SUD/06011	6/00	2007-02-01	2007-07-31
SAYED, Mr WA	EGY/06034	9/26	2007-03-06	2008-03-05
ALI, Ms S	SUD/04023	6/00	2007-04-05	2007-10-04
YESMIN, Ms F	BGD/06027	3/00	2007-05-01	2007-07-31
NURHAYATI, Ms S	INS/06044	4/00	2007-05-01	2007-08-31
ABIDIN, Mr RA	INS/06054	4/00	2007-05-01	2007-08-31
SOLIS ECHEVERRIA, Mr E	MEX/06016	1/00	2007-08-01	2007-08-31
GARCIA MARTINEZ, Mr V	MEX/07005	1/00	2007-09-03	2007-10-02
DEGEFA, Mr AY	ETH/07010	1/00	2007-11-01	2007-11-30
DEMISSIE, Mr WS	ETH/07007	1/00	2007-11-01	2007-11-11
LEMMA, Mr NZ	ETH/07006	1/00	2007-11-01	2007-11-16
GULMMA, Mr SY	ETH/07011	0/11	2007-11-01	2007-11-16
NAMBIRO, Mr CJO	KEN/07005	0/16	2007-12-17	2007-12-21
Scientific Visitors				
GABINDADDE-MUSOKE, Mr D	UGA/07007V	0/01	2007-05-17	2007-05-17
RWAMIRAMA KANYONTOLE, Mr B	UGA/07006V	0/01	2007-05-17	2007-05-17
LAVI, Ms E	ISR/08001V	0/10	2007-12-03	2007-12-14

5.5. Coordinated Research Projects and Technical Cooperation Projects

CRP Title	Scientific Secretary
Application of established molecular technologies to improve the effectiveness of SIT (2003-2008)	Gerald Franz
Development of mass rearing in support of *Anastrepha* and *Bactrocera* fruit fly SIT (2004-2008)	Carlos Caceres
Enabling technologies for the expansion of SIT for New World and Old World screwworm flies (2001-2006)	Alan Robinson
Product and process quality control for standardization of tsetse mass production, sterilization and SIT release (2003-2007)	Andrew Parker
Improving SIT for tsetse flies through research on their symbionts and pathogens (2007-2013)	Adly Abd-Alla
Mass rearing and pre-release biology of *An. arabiensis* (2005-2009)	Colin Malcolm
TCP Title	**Technical Officer**
Integrating Sterile Insect Technique for Tsetse Eradication	Andrew Parker
Integrated Area-Wide Tsetse and Trypanosomosis Management in Lambwe Valley	Andrew Parker
Sterile Insect Technique for Area-wide Tsetse and Trypanosomosis Management	Andrew Parker
Preparations for the Creation of a Zone Free of G. Brevipalpis and G. Austeni	Andrew Parker

5.6. Abbreviations

ARS	Agricultural Research Service
ATG	Adenine-Thiamine-Guanine (start codon)
AW-IPM	Area-Wide Integrated Pest Management
CRP	Coordinated Research Project
CTC	Chlortetracycline
DNA	Deoxyribonucleic Acid
DTS	Dominant Temperature-Sensitive
DUR	Dose Uniformity Ratio
EA-IRMS	Elemental Analyser - Isotope Ratio Mass Spectrometer
EGFP	Enhanced Green Fluorescent Protein
FAO	Food & Agriculture Organization
GIS	Geographical Information Systems
GPS	Global Positioning Systems
GSS	Genetic Sexing Strain
IAEA	International Atomic Energy Agency
ISI	Index of Sexual Isolation
ORF	Open Reading Frame
PAGE	Pulse Field Agarose Gel Elecrophoresis
PCR	Polymerase Chain Reaction
PM	Proportion of Mating
PMMA	Polymethyl Mmethacrylate (Perspex®, Plexiglas®, Lucite®)
QC	Quality Control
RFLP	Random Fragment Length Polymorphism
RNA	Ribonucleic Acid
RS	Remote Sensing
RSI	Relative Sterility Index
SGH	Salivary Gland Hypertrophy
SGHV	Salivary Gland Hypertrophy Virus
SIT	Sterile Insect Technique
SKNIR	Single Kernal Near Infrared Spectrometer
TCP	Technical Cooperation Projects
TPU	Tsetse Production Unit
USDA	United States Department of Agriculture

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