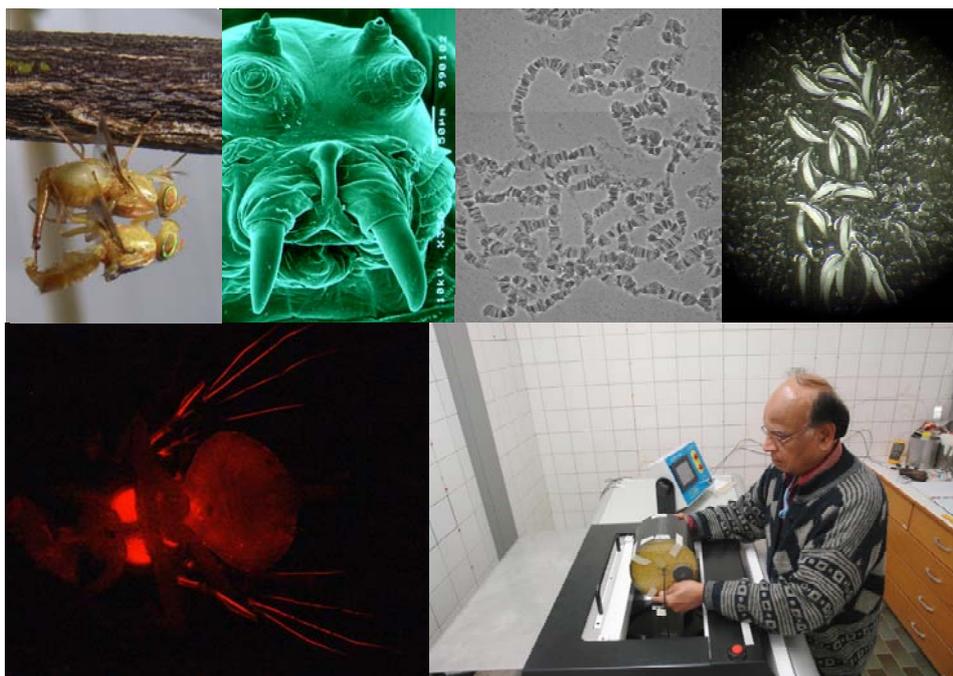**Joint FAO/IAEA Programme**
Nuclear Techniques in Food and Agriculture

Entomology Unit

Activities Report

2008

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

Executive Summary

The presence of the salivary gland hypertrophy virus in the colonies of Glossina pallidipes *at the Unit and in the Kaliti facility in Addis Ababa, Ethiopia not only remains a cause for concern, but is a permanent threat to the effectiveness of the tsetse AW-IPM programme in Ethiopia that has a prominent SIT component. Both colonies experienced serious problems in 2008, which may be related to the presence of the virus. Following the very successful genomic analysis of the virus, its secuence has now been published. This information is now being used in efforts to develop effective ways to manage the virus in the colonies. Studies on the effect of the virus on productivity in the colony indicated that all males that showed salivary gland hypertrophy produced no offspring. A portion of symptomatic females produced offspring but these were likewise symptomatic and were completely sterile. Work has likewise been initiated on the effect of antiviral drugs on the virus, on the use of antibodies and gene knockout using RNA interference, and a proposal has been submitted by the International Committee of Taxonomy of Viruses to the Archives of Virology to classify the virus into a new virus family.*

Work continued on the custom-made X-ray machine, which received a new X-ray tube in the beginning of 2008. The machine performed very satisfactorily throughout the year allowing a series of dosimetry measurements that resulted in some small changes in the design of the radiation canisters but confirming that the machine can deliver the required radiation dose to the required volumes of pupae for use in SIT programmes. In addition to the physical dosimetry, experiments were carried out to assess the biological effectiveness of X-rays in comparison with gamma rays from a standard gamma cell. Basic radiation biology studies were carried out on several species of fruit fly, together with field cage evaluations of mating competitiveness. From these initial studies it appears that there are no significant differences between the two types of radiation source as to their suitability for use in SIT programmes.

In Sudan, the counterparts have made good progress with the planning of their pilot programme to assess the feasibility of using the SIT against the mosquito Anopheles arabiensis. *This has required the immediate development of basic implementable mosquito production and release methods. Due to the uniqueness of the project in Sudan and the fact that there is little equipment available as an off-the-shelf resource, priority was given in the Unit to designing and developing several prototype devices and procedures such as a larval rearing tray, prototype racks for stacking the larval trays, adult cages and a device to separate the larvae from the pupae. Considerable efforts were given to understanding larval responses to mass-culture conditions, methods for storage of adults, pupae and eggs, and experiments were conducted to develop a larval diet that is suitable for mass-rearing.*

The olive fruit fly Bactrocera oleae *is the key pest in all regions cultivating olives in the Old World and it has recently invaded California and Mexico. Between 1970 and 1985 various unsuccessful attempts were made to develop the SIT package for this pest, and as a result, large field programmes were never developed or implemented. In the ensuing years the economics related to olive production have changed, as has the availability of acceptable methods of control. Following numerous requests from Member States to develop alternative control tactics that are friendly to the environment, the Unit re-initiated work on the olive fly in 2005. This year much progress has been made in terms of rearing technology. Cheaper larval diets have been developed together with improved cages for egg production. In addition, new egg handling protocols have been introduced resulting in significant increases in pupal production. The importance of fresh egg yolk in the diet of olive fly was confirmed.*

Studies with male melon flies Bactrocera cucurbitae *indicated that exposure to methoprene and addition of protein to the diet accelerated their sexual maturity and significantly enhanced their contribution to lek initiation, lek participation and their dominance in the leks, which resulted in overall improved mating performance.*

Work was initiated with the invasive fruit fly species Bactrocera dorsalis *and* Bactrocera invadens, *which are difficult to distinguish from each other based on morphological and behavioural characteristics. A series of hybridization experiments were conducted to assess their sexual compatibility. These experiments indicated that mating occurred readily under forced laboratory conditions and that viable eggs were produced resulting in adult F_1 offspring. Further experiments will focus on non-forced choice mating experiments and field cage tests.*

Standard polythene chromosome maps were developed for the Mexican fruit fly Anastrepha ludens *and the performance in the laboratory of a transgenic sexing strain of the Mediterranean fruit fly* Ceratitis capitata *(produced by the company Oxitec) was compared with a wild type strain and non-transgenic sexing strains.*

Earlier research indicated that stable isotopes could be a valuable tool to assess the origin of trapped insects and could be used to study dispersal patterns of released insects. Work continued in the Unit and focused on possibilities to assess mating status and sperm transfer in the Mediterranean fruit fly and the South American fruit fly, and comparisons were made between the isotopic signal of wild and reared tsetse fly Glossina pallidipes *and of wild and reared pink bollworms* Pectinophora gossypiella.

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1. PROGRAMMATIC AND UNIT OBJECTIVES

The vision and goals of the Insect Pest Control sub-programme are to increase food security and to alleviate hunger through the development of the Sterile Insect Technique for area-wide integrated pest management (AW-IPM) programmes to control key insect pests of agriculture and human health. The sub-programme achieves these goals through:

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

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

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

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

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3. RESEARCH AND DEVELOPMENT ACTIVITIES

3.1. Tsetse Rearing and Virus Analysis

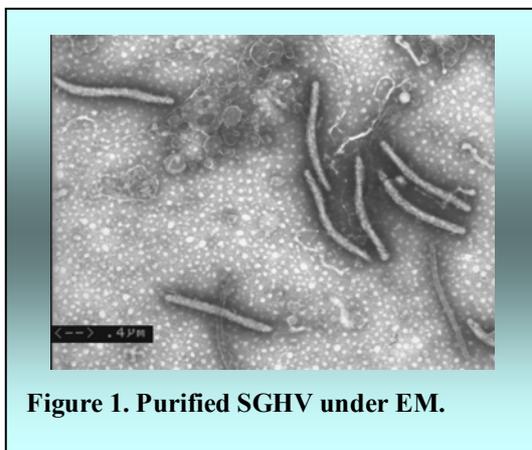#### 3.1.1. *Salivary Gland Hypertrophy Virus Studies*

Figure 1. Purified SGHV under EM.

As reported in previous Activities Reports some tsetse species carry a virus (**Figure 1**) that, in a certain proportion of individuals leads to salivary gland hypertrophy (SGH) and these individuals also show reproductive abnormalities. In natural populations the prevalence of the virus is low (0.5-5%) based on salivary gland dissection and in a colony of *G. pallidipes* that originated from Uganda and that is maintained in the Unit, the frequency of SGH ranges from 4-10%. However, PCR analysis has confirmed that virus prevalence is almost 100% in the laboratory colony. The virus was also detected in samples of *G. pallidipes* from the colony maintained at the Kaliti facility in Ethiopia. Due to the negative impact of the virus on colony productivity under certain stressful conditions it is important to understand more about the virus with the goal to develop a strategy for its management. The most effective way to begin this study is to understand more about the virus in terms of its taxonomy 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. The complete genome sequence was determined and published. The information obtained by the genome sequence provides opportunities for the use of quantitative PCR (qPCR) and RNA interference (RNAi) technology as potential tools in the development of virus management protocols. In addition, work on the effect of some antiviral drugs to suppress viral replication is in progress. Experiments to understand the virus transmission in the colony, and effect of stress condition were also carried out.

3.1.1.1. *Virus transmission in the* Glossina pallidipes *colony*

To analyze the impact of the virus on the productivity of the flies, 400 couples of tsetse flies were mated in individual tubes. Two days after mating the males were separated, dissected, and the state of salivary gland recorded. The females were maintained until they had produced the third pupa or for 60 days, then the females were dissected and the salivary gland state was recorded. The F_1 pupae were incubated individually until emergence. Based on the state of the salivary gland of the parent the pupae were divided into three groups: (1) both parents with normal salivary glands, (2) parental males with normal salivary glands and parental females with hypertrophied glands, and (3) parental males with hypertrophied glands and parental females with normal glands. After emergence of the F_1 pupae, flies were mated within each group and following mating the males were dissected as previously described and the females were maintained until they produced a third pupae or for 60 days, then dissected. The results, presented in **Figure 2** show that no hypertrophied salivary glands were detected in the progeny of group 1. In contrast, in the progeny of group 2, all females and 62.5% of the males had hypertrophied salivary glands. Also, the productivity in the progeny of group 1 was 0.92 pupae per initial female, while group 2 produced no progeny. In group 3 very few progeny

were produced. These results indicate that almost all males with salivary gland hypertrophy (SGH) produced no progeny but that females with SGH can produce progeny but their progeny will have SGH and became completely sterile in F_1.

3.1.1.2. *Virus copy number in various tissues in symptomatic and asymptomatic males*

To explore the relationship between the enlarged salivary gland in the flies and the virus copy numbers, a qPCR test has been optimised and used. Symptomatic infected males were selected based on external observation and confirmed by dissection to isolate the salivary glands. Twelve males with a symptomatic infection and an equal number of males with asymptomatic infection were selected. For each male the right intermediate leg was excised with sterile scissors, the salivary glands were collected and the remaining whole body was kept

Figure 2. Impact of SGH on *G. pallidipes* productivity.

separately in a microtube. The total DNA was extracted from all samples. The DNA extracted from excised leg and salivary glands were diluted 1:100 whereas DNA extracted from the whole body was diluted 1:1000. Using the above calibration, the virus copy number was determined in the total DNA extracted from a single intermediate leg, a pair of salivary glands and remains of the body of 12 symptomatic and 12 asymptomatic flies. In asymptomatic males, an average of 1.68E+5, 2.05E+5 and 1.07E+7 virus copy number was estimated in legs, salivary glands and fly body, respectively. In symptomatic males the copy number was significantly higher with averages of 1.34E+7, 1.42E+10 and 1.5E+9, respectively **(Figure 3)**.

Figure 3. Virus copy number (log) between asymptomatic and symptomatic male *G. pallidipes*. DNA was extracted from a single excised middle leg (Leg), salivary glands (SG) and whole fly (body).

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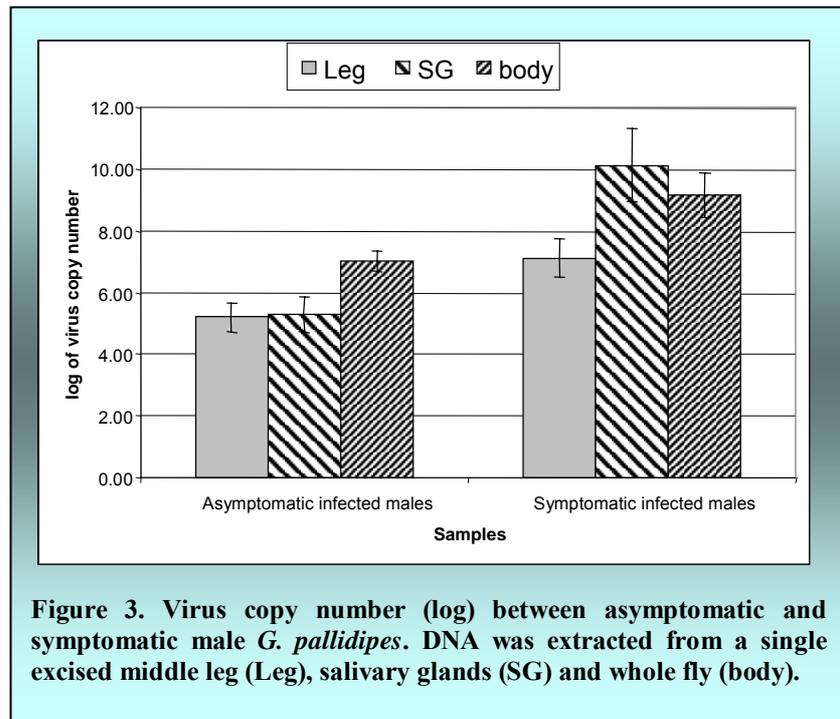
3.1.1.3.   Quantification of virus copy number in females and their progeny

Figure 4. Two dimensional scatter plot of GpSGHV log copy in symptomatic and asymptomatic infected mothers versus their pupae. Filled and open circles represent symptomatic and asymptomatic infected mothers, respectively.
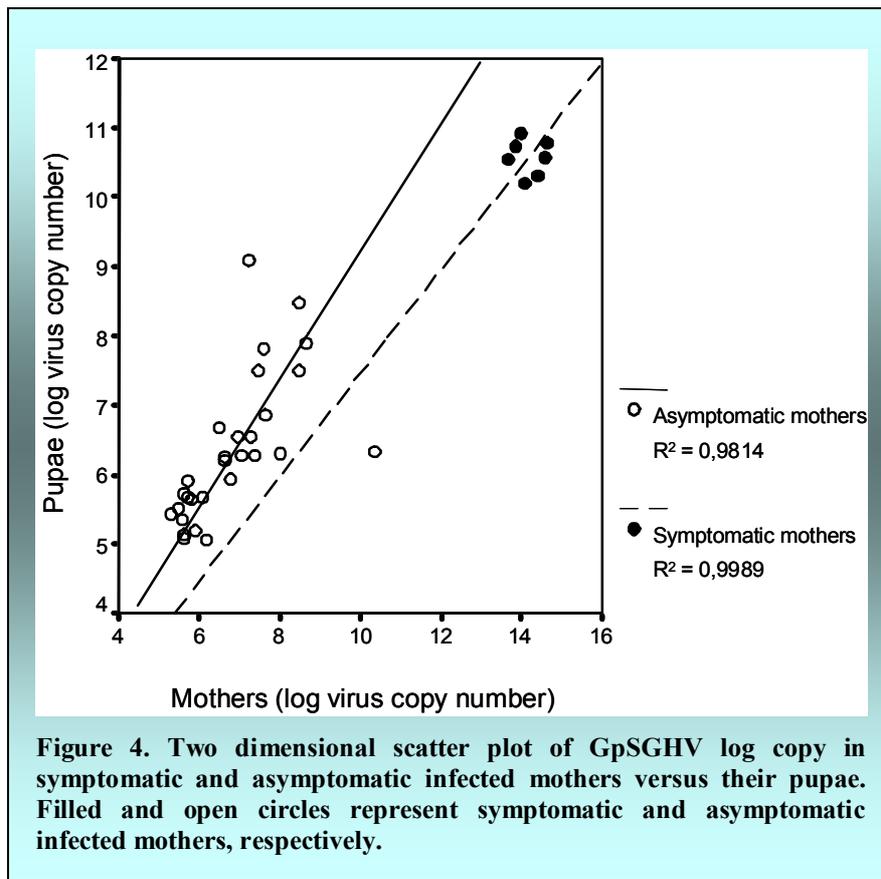
To determine the relationship between the virus copy number in pupae and in their mothers, 36 pregnant females were selected from the colony and placed in individual cylindrical tubes (4 x 6.5 cm) on sterile Petri dishes until the larva was deposited. Pupation occurred within 10-15 min post-larviposition. The female was then dissected to check the status of the salivary glands, and both the pupa and the mother were processed for total DNA extraction. DNA was diluted to 1:1000 and used for qPCR. The results presented in **Figure 4** show a clear correlation between the virus copy number in mother and offspring and two distinct groups of data points were observed. The group with low virus copy number in both mothers and progeny corresponded to asymptomatic females (verified by dissection), whereas the group with high copy number all corresponded to symptomatic females. There was a significantly positive correlation between virus copy number in mothers and progeny in asymptomatic flies and symptomatic flies (r = 0.930, *P* < 0.0001).

3.1.1.4.   Effects of stress on salivary gland hypertrophy

To collect more information about the biology of the tsetse virus, we analyzed the impact of high temperature in combination with fly density in the holding cages on the prevalence of SGH. Two temperatures, 25ºC and 30ºC, and seven fly densities were tested. Fly density per cage was positively correlated with mortality and negatively correlated with productivity and with temperature. No correlation was observed between the rate of hypertrophy, fly density per cage, and temperature (**Figure 5**).

Figure 5. Impact of fly density and temperature on the prevalence of SGH in tsetse flies colony.
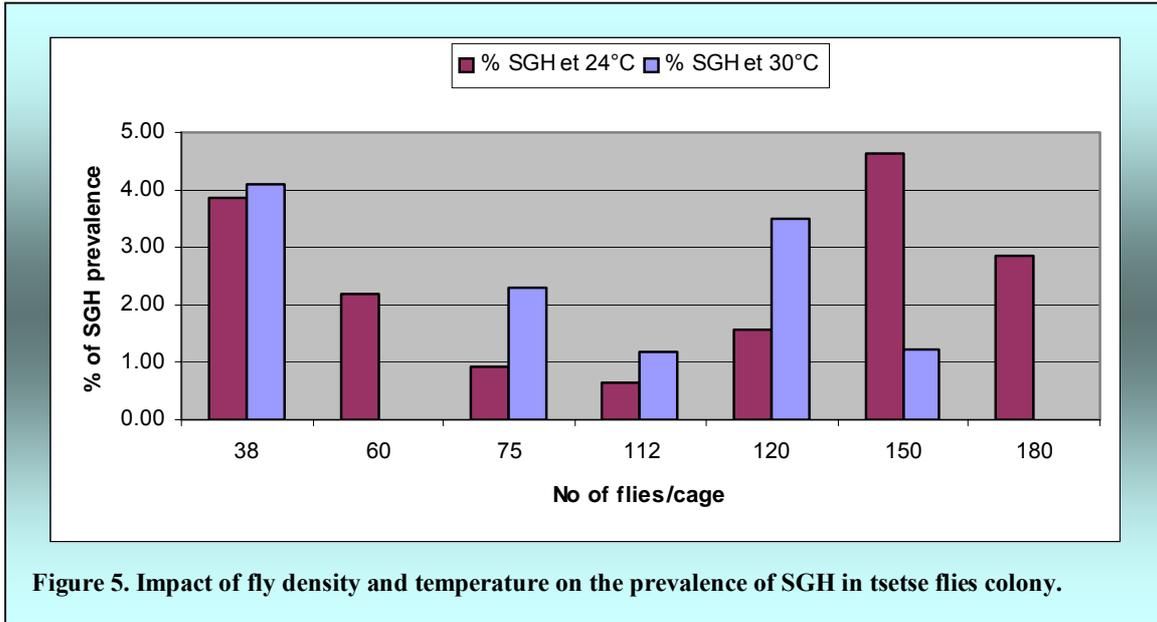
3.1.1.5.　　Impact of antiviral drugs

As reported previously the impact of two antiviral drugs on virus replication was analyzed by qPCR. Only a slight decrease in the virus copy number was found with the antiviral drug treatment so no real conclusions could be drawn. The experiment was repeated with five concentrations of each antiviral drug to determine the maximum concentration we can use without affecting the productivity and the mortality rate in the flies. We have focused on the impact of the antiviral treatment on the fly mortality and productivity in the parental generation, and are continuing the experiment to analyze the impact on the subsequent generations. Significant reductions in the fly mortality and acceptable rates of pupal production (**Figure 6**) were observed

Figure 6. Impact of antiviral treatment on mortality and productivity of *G. pallidipes*.

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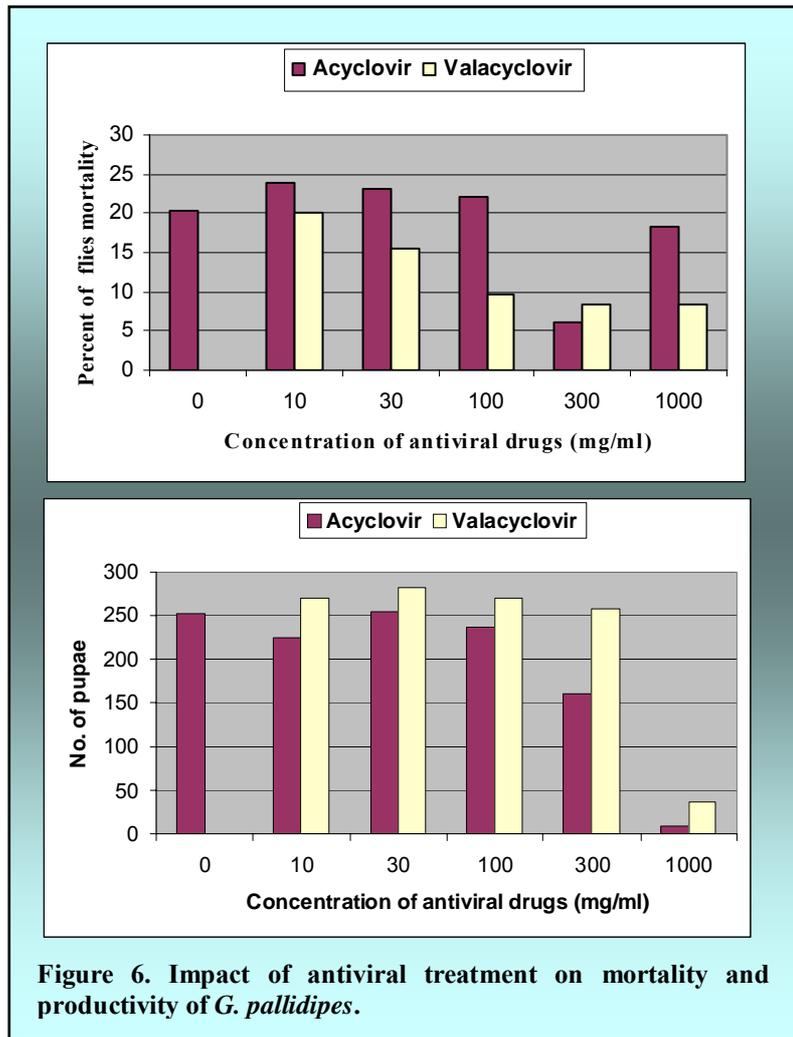
with several antiviral drug treatments. Further analyses to assess the virus load in the different treatments were carried out using qPCR. Eight flies (two males and six females) from each treatment (with three replicates from each treatment) were used for qPCR analysis. Total DNA was extracted from each fly individually for qPCR analysis. The results did not show a clear correlation between the SGH prevalence and the acyclovir treatments (**Figure 7**). This result could be explained by the difference of the initial number of flies with high virus copy number in each treatment. As we cannot start the experiment with flies having similar levels of virus load, another method to analyze the results needs to be investigated. Based on the results of the F_1 a reduced number of promising treatments were selected to continue the work in the subsequent generations. The mortality and the productivity record in the F_1 generation are presented in **Figure 8**. This experiment will be followed until the F_4 generation.
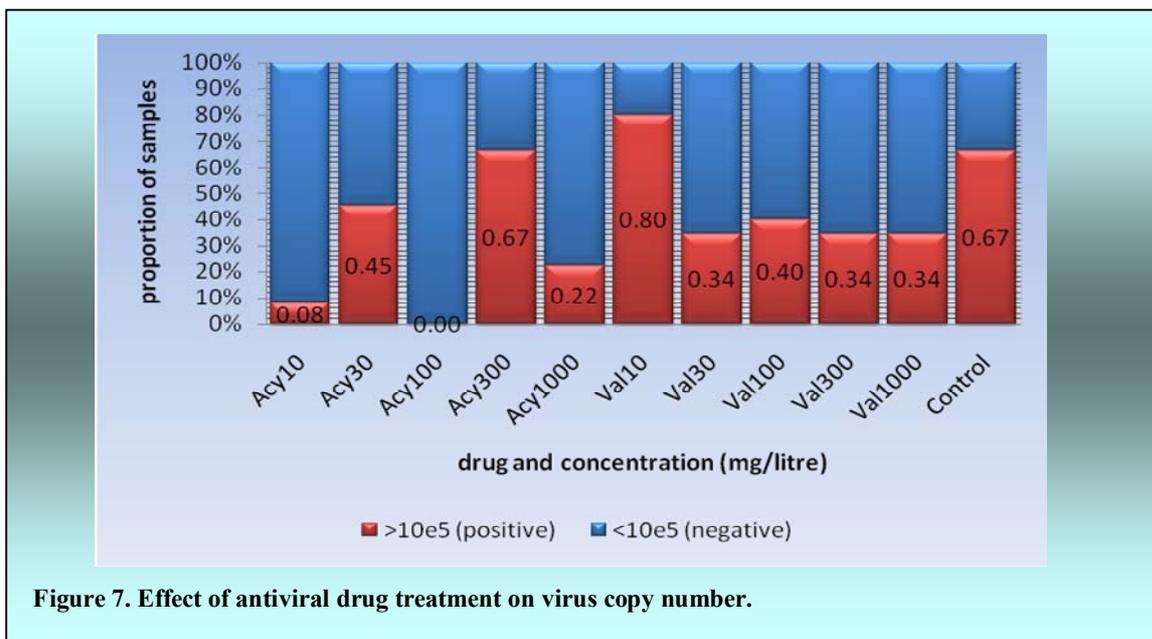
Figure 7. Effect of antiviral drug treatment on virus copy number.
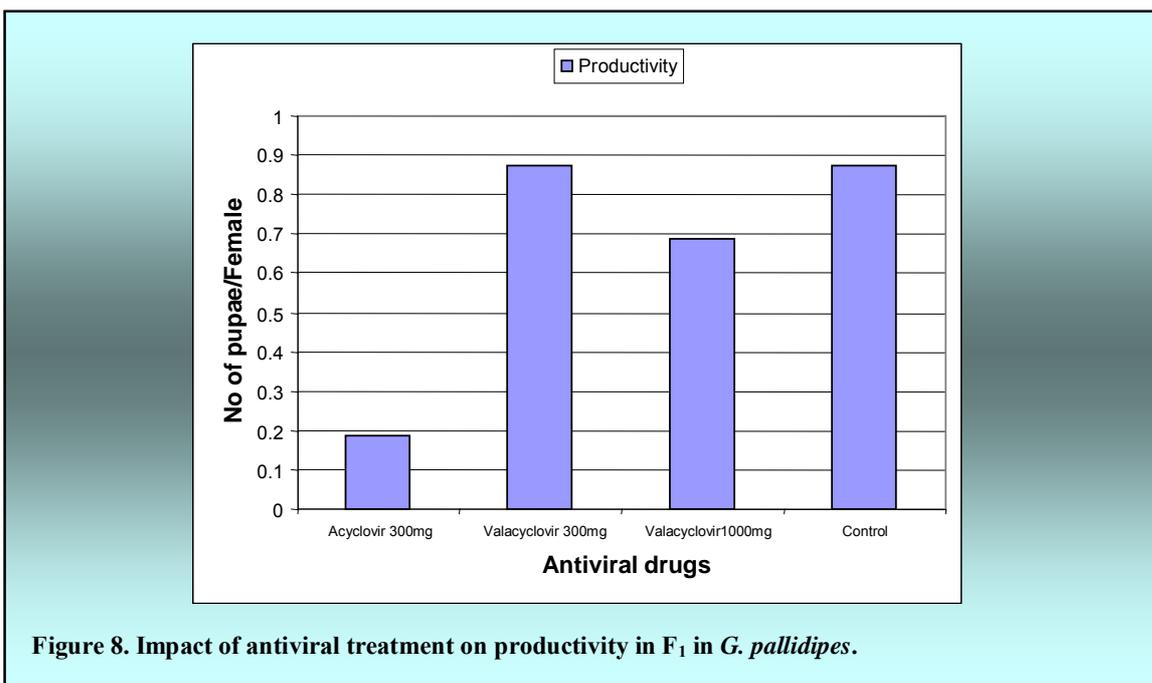
Figure 8. Impact of antiviral treatment on productivity in F_1 in *G. pallidipes*.
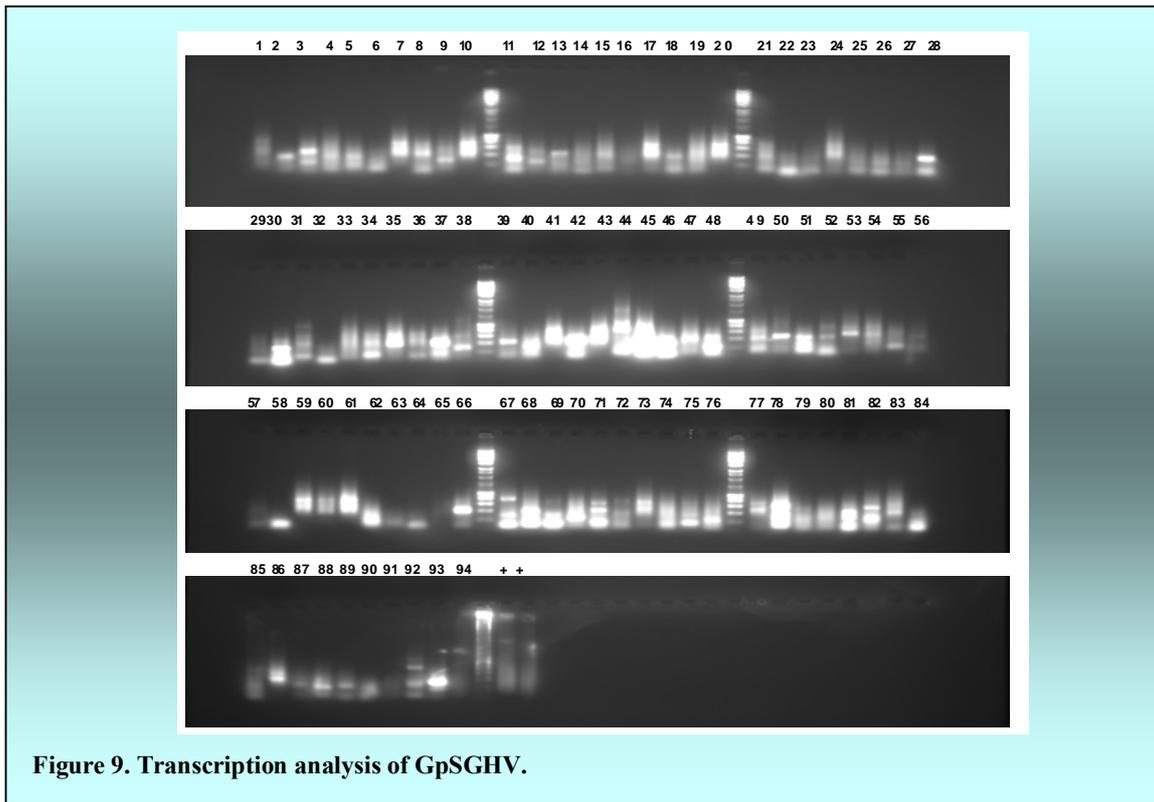
Figure 9. Transcription analysis of GpSGHV.

3.1.1.6.   *Transcription analysis*

After completing the sequence of the genome of GpSGHV, 160 open reading frames (ORF) were predicted using genome analysis software. To obtain more information about the virus at the molecular level and to proceed to the RNAi technology to find a way to reduce or stop virus replication it was essential to analyze virus transcription to confirm the predicted ORFs and to have an idea about the transcription level of each ORF. We have analyzed the virus transcription by extracting the mRNA from hypertrophied glands followed by DNase treatment. After confirming the absence of DNA traces, cDNA was generated by reverse transcription using poly A and subsequently two specific primers for each ORF were used to perform PCR amplification. The results presented in **Figure 9** show the presence of mRNA for most of the predicted ORFs.

3.1.1.7.   *RNAi experiment*

RNAi technology can be used to reduce the expression of specific genes in animals, plants and many invertebrates. Development of RNAi technology, to reduce or stop the virus infection in tsetse colonies, was proposed as a virus control strategy in addition to the antiviral drugs and antibody immunization approaches. Arbitrarily, one gene was selected to test the application of RNAi on tsetse flies. The N-terminal, the C-terminal and the total sequence of the selected gene were cloned in a plasmid vector that was used to transform *E. coli*. The impact of adding the non transformed bacteria to the tsetse blood diet on the productivity and survival of flies were tested using different concentration of live and killed bacteria **(Figure 10).** Based on these results, transformed bacteria with different constructs of the selected gene were used with the highest allowable concentration of killed bacteria to feed tsetse fly females for 60 days or until they produced their third pupa. Fly mortality and

productivity were recorded and are presented in **Figure 11**. The impact of the RNAi treatment on the virus copy number will be measured using qPCR and the experiment will be continued into the F_1 generation.

Figure 10. Effect of *E. coli* bacteria on productivity and mortality of *G. pallidipes*. Control: normal blood diet, BP0: blood diet containing bacteria with no plasmid, BP1: blood diet with bacteria containing plasmid 1, etc.

Figure 11. Effect of RNAi treatment on productivity and mortality of *G. pallidipes*.

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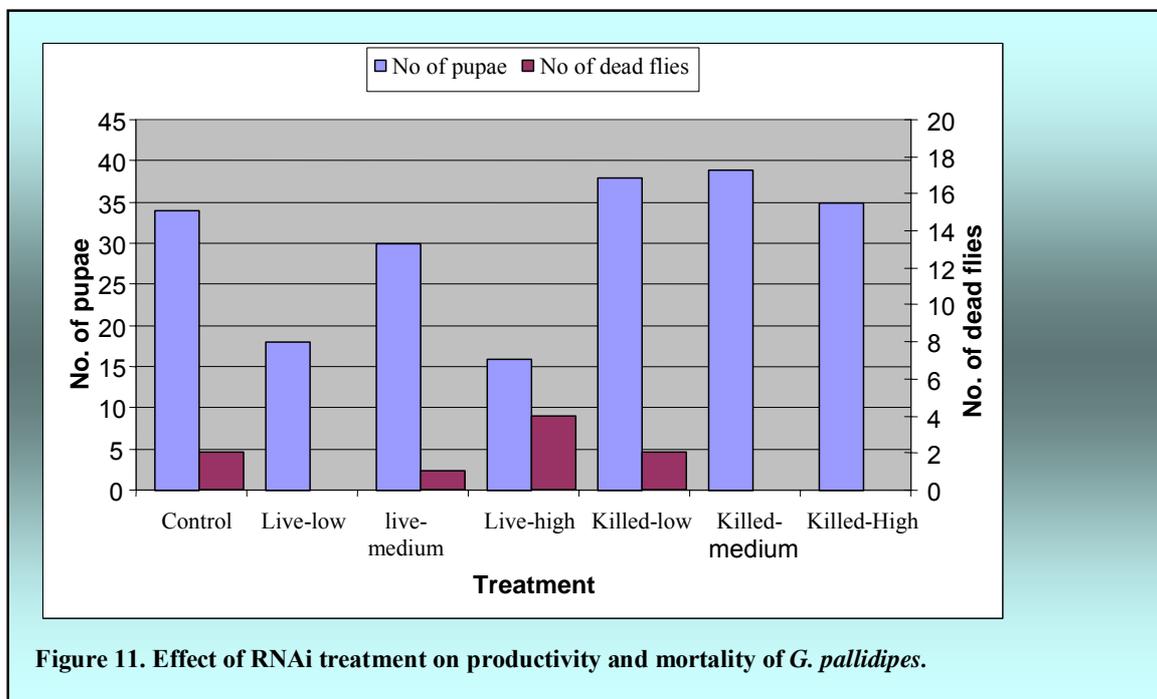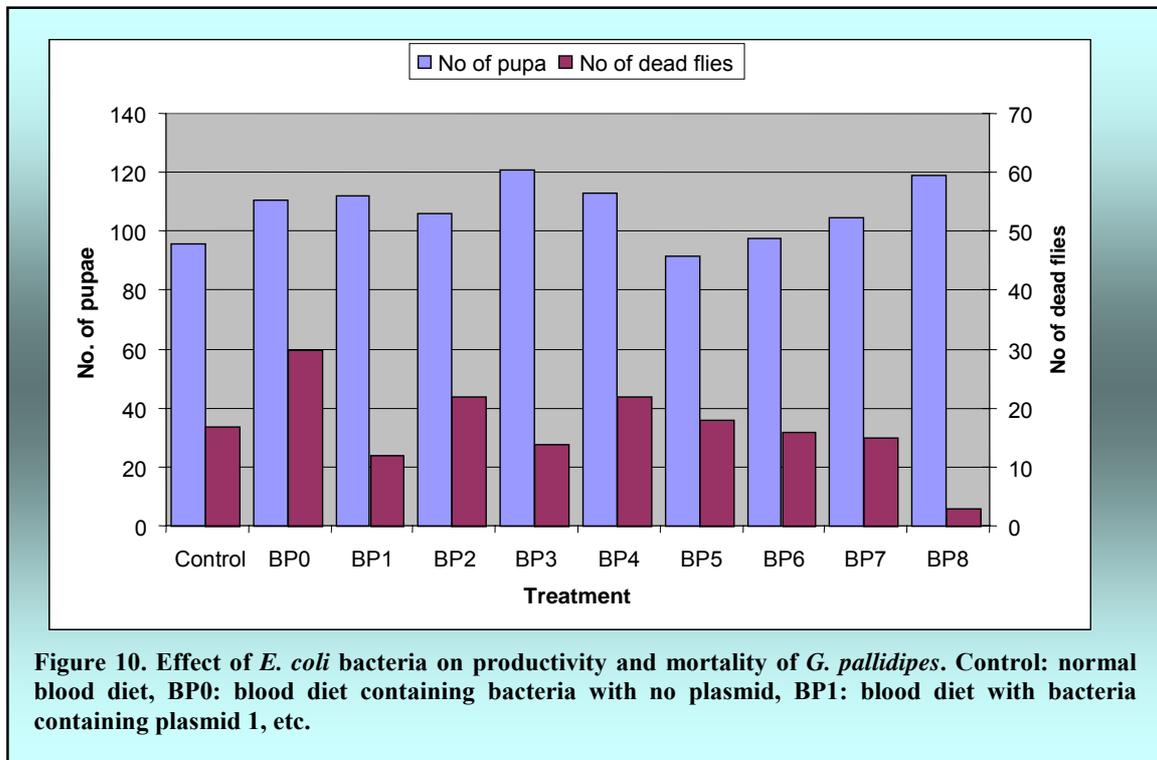
3.1.1.8.   *Virus classification*

Attempts to classify the GpSGHV are ongoing. A study group composed of virus experts has been established by the International Committee on Taxonomy of Viruses. A proposal for classification of the virus has been submitted to the Archives of Virology. The proposed classification generates a new virus family *Hytrosaviridae* with three virus species. These species include the tsetse fly *G. pallidipes* salivary gland hypertrophy virus (GpSGHV), the house fly *Musca domestica* salivary gland hypertrophy virus (MdSGHV) and the narcissus bulb fly *Meredon equestris* salivary gland hypertrophy virus (MeSGHV).

3.1.2. *Experimental Modification of Male Genitalia and Effects on Female Choice*

Dr Daniel Briceño, a consultant from the University of Costa Rica, visited the Entomology Unit for the whole of 2008. Dr Briceño has studied the mating behaviour of tephritid fruit flies for many years. Following work on tsetse mating behaviour under a Coordinated Research Project, he was invited to the Unit to expand his study of the mating behaviour of tsetse flies and in particular the significance of specific genitalic structures and their effect on female reproductive behaviour and physiology. This work will contribute to improving quality control protocols for sterile male tsetse.

One of the most sweeping of all evolutionary patterns in morphology is for male genitalia, in species with internal insemination, to diverge especially rapidly compared with other body structures. One hypothesis to explain this unusual pattern is that male genitalia function as courtship devices, and diverge rapidly because they are under sexual selection by cryptic female choice. Sexual selection by cryptic female choice occurs when the females of a species modulate reproductive processes under their control that occur after copulation has begun so as to favour the potential paternity of some males over that of others, and this bias is correlated with differences among males with respect to particular traits (such as genital morphology). A powerful technique for checking the possible influence of a male's genital traits on paternity is to experimentally modify his genitalia or else the receptors in the area of the female that they contact during copulation, and then determine the effects on female reproductive processes that could result in cryptic female choice. The present study of the tsetse flies *G. pallidipes* and *G. morsitans* constitutes the most diverse set of experimental alterations of male genital form and the possible female perception of their form yet performed, and reports the most varied effects ever seen on female reproductive responses to genital modifications.

In *Glossina* copulation lasts 30 min or more, and a spermatophore is transferred in approximately the last 30s. Stimulation associated with copulation induces the female to ovulate a single egg, which is fertilized, probably in the oviduct, then hatches in the female's "uterus". It has been shown that in *G. morsitans* the stimuli that induce ovulation are not derived from transfer of sperm, deposition of the spermatophore in the female, secretions of the male's testes, accessory glands or ejaculatory ducts, or from humeral factors from the spermathecae of inseminated females. Instead, mechanical stimulation received during copulation itself seems to induce ovulation, but it has yet to be determined what stimuli these might be.

A second response of female *G. morsitans* to copulation is a diminished receptivity to additional mating attempts by males, and it has been shown that undetermined mechanical stimuli during copulation (as well as male accessory gland substances and distension of the
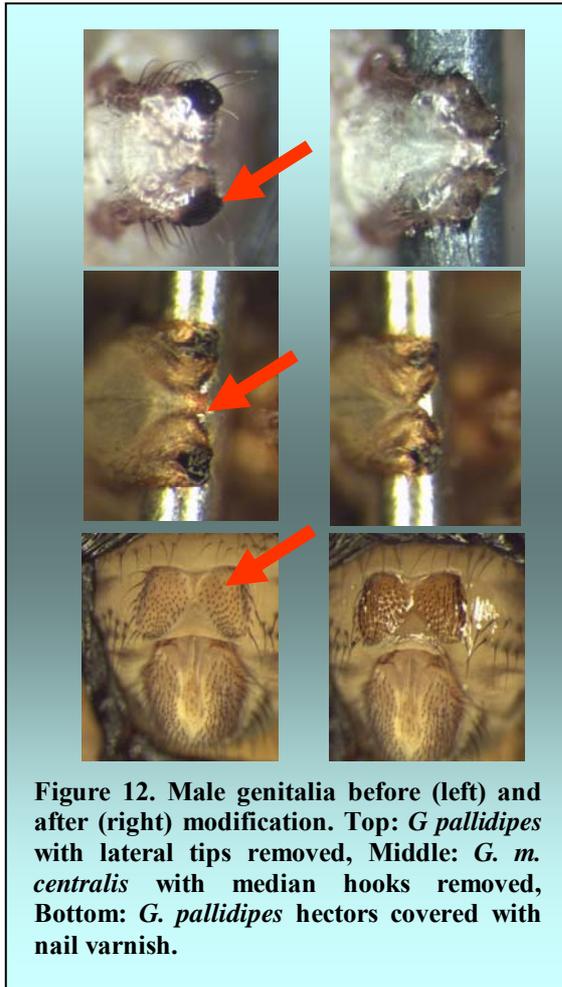
Figure 12. Male genitalia before (left) and after (right) modification. Top: *G pallidipes* with lateral tips removed, Middle: *G. m. centralis* with median hooks removed, Bottom: *G. pallidipes* hectors covered with nail varnish.

uterus) also trigger this female response. There is less direct evidence that females may also exercise some control over intromission, and over sperm transfer.

There are many candidate stimuli associated with copulation that might induce these female responses. Males of *G. pallidipes* perform energetic and sustained courtship behaviour during copulation, and may stimulate females during copulation with six different types of behaviour patterns, including sounds and potential visual stimuli from their wings, and stylized rubbing on different parts of the female with all three pairs of legs. In addition, males also move some portions of their genitalia with vigorous, rhythmic, sustained squeezing movements. Several male genital structures contact the female, and six of these have morphological modifications that appear to be designed to stimulate the female. Copulation behaviour in *G. morsitans* is similar in that males perform apparent courtship behaviour with both their genitalia and their legs and wings.

Experimental alteration of the form of two species-specific male genital structures that remain on the outside of the female during copulation reduced three potential cryptic female choice responses in *G. pallidipes*: sperm storage, ovulation, and female resistance to remating. The possibility that these effects were due to changes in male behaviour rather than changes in the stimuli received by the female from his altered genitalia was ruled out by two sets of experiments: similar effects on females were obtained when the female sensory abilities at the sites that are contacted by these male structures during copulation were extinguished or severely altered; and no change was seen in female behaviour when the male's lack of stimulation during copulation that resulted from experimental alteration (**Figure 12**) was mimicked by extinguishing or severely altering the stimuli he received from this portion of his genitalia.

Deductions from previous studies of behaviour, morphology, and physiology that suggested that these structures function to stimulate females to gain paternity were thus confirmed. This is the most complete direct confirmation of the hypothesis that sexual selection by cryptic female choice has been responsible for the rapid divergent evolution that is typical of male genitalia.

3.1.3.　X-Ray Irradiator: Reliability and Dosimetry

Much effort was made during the year on enhancing the performance of the Rad Source RS2400 X-ray irradiator. The aim was to solve the two problems that were identified last year

(**Activities Report 2007**), namely, reliability of the irradiator and dose distribution in the canister.

Earlier in the year, Rad Source replaced the first X-ray tube with a modified tube which had better cooling water flow around the tube as well as an improved method for installation. This tube can now be operated at up to 45 mA rather than 35 mA. This increases the power output of the tube by about 28% with a corresponding increase in the dose rate. This tube has been in operation for almost a year without any problems, which shows that its reliability has significantly increased.

Another characteristic that needed improvement was the dose distribution in the canister. The aim was to achieve a dose uniformity ratio (DUR) of about 1.3. Last year, this value could be achieved only by significantly reducing the size of the canister, which is not a preferable option. It was realized that one of the causes for the poor dose uniformity was that X-rays contain a wide energy spread, from about 30 to 150 keV, unlike cobalt-60 gamma radiation. Thus, in the case of an X-ray irradiator, the low-energy X-rays (photons) deliver high dose near the entrance surface of the canister, resulting in a high value of DUR. Thus, the DUR could be decreased by 'hardening' the X-ray energy spectrum by removing the low-energy photons before they reach the canister (pupae). A metal jacket around the canister can easily absorb these photons. **Figure 13** shows the effect of 1mm brass and 0.5 mm steel on the dose rate in the canister centre and the dose distribution along the canister diameter. The canister wall is made of 2 mm carbon fibre. The DUR for the bare canister (without metal surround) was 1.21 (considering only the direction along the diameter). Adding 1-mm brass made this ratio almost unity. However, it also reduced the centre dose rate by about 70%. On the other hand, surrounding the canister with 0.5mm steel yielded DUR of about 1.06 and the dose rate reduction was only 40% as compared to that for the bare canister. Thus, 0.5 mm steel is the recommended filter around the canister for hardening the X-ray spectrum in the RS2400.

Figure 13. Effect of different filtration on the radial dose uniformity in an irradiation canister. Carbon = carbon fibre canister without additional filtration; steel = canister with a 0.5 mm steel filter covering the whole of the curved surface of the canister; brass = canister with a 1.0 mm brass filter covering the whole of the curved surface. Steel and brass are substantially equivalent in terms of filtration and absorption of X radiation.

Figures 14 and **15** show the axial and radial (along the diameter) dose distributions for the final geometry of the canister. These measurements were made with all five canisters full of instant rice (which behaves very similar to pupae under irradiation). Two separate runs were made; one for axial dose distribution and the other for radial dose distribution. For the axial

Figure 14. Axial dose distribution measured at three positions, one in the centre-line (central axis of the canister), the other two at diametrically opposed positions on the inside of the canister wall corresponding to the positions closest to and furthest from the X ray tube when the canister is first loaded into the irradiator.

Figure 15. Dose variation across two perpendicular diameters in the centre of the irradiation canister. Carbon fibre canister with 0.5 mm steel filter as in Figure 14.

run, three 20 cm long Gafchromic films were placed within the canister along its length: one in the centre (along the axis) and two near the periphery (laid on the curved surface). For the radial run, two 18 cm long Gafchromic films were placed perpendicular to each other and both along the diameter going through the centre of the canister. For both runs, the irradiator was operated at 150 kV/17.5 mA for 20 min, with a rotation speed of 5 rpm. Low current was selected so that there are several revolutions of the canisters with the result that the dose distribution is not significantly affected by the last non-complete revolution. For these measurements, the canister was 20 cm long. To achieve a DUR of 1.3, the canister was shortened to 15 cm, yielding a volume of just over 3.7L. Thus 18L of pupae can be irradiated per batch.

The dose rate in the centre of the canister filled with instant rice is about 14 Gy/min. When the canister is filled with fruit fly pupae the dose rate decreases by 6%. This dose rate was measured by a Farmer type (0.18-cc) ionization chamber which was calibrated in the energy range of 40 keV to 1.33 MeV with traceability to the National Institute of Standards and Technology (NIST) in the USA. Such a reference dosimetry system is very essential for the calibration of the routine Gafchromic dosimetry system. Data on the effects of X-rays on fruit flies are presented in Section 4.

3.1.4. Introducing LabPal

During the year the FAO/IAEA Agriculture & Biotechnology Laboratory has been updating and expanding its laboratory information management system, LabPal. The Entomology Unit is participating in this through the assembly of a large library of reference biological material, both whole insects and extracted DNA. This library has expanded rapidly over the past year and there was an urgent need to develop an information management module for this. The Unit, therefore, in conjunction with the developers, has designed a new module within LabPal called Bio Resources to address this need. The new module is accessed from the Entomology Unit main page (**Figure 16**) in LabPal. It is arranged in sections for incoming samples, internal sub-samples (e.g. for a sample divided for different PCR runs), external sub-samples (for material sent to another laboratory), storage and data export as well as a data entry

Figure 16. Main page showing selection criteria for filtering the entries to locate the sample required.

module (**Figure 17**) for direct entry of data or for importing from a spreadsheet. The record for each sample contains full data on species, origin, exact location (using GPS), number of individuals in the sample, storage method, and storage location under a unique identification number. The identification number is used to generate a two dimensional bar code for labelling tubes and vials, and storage boxes, racks, freezers and rooms are also bar coded (**Figure 18**). The database is relational so can be linked to individual wells in, for example, a 96-well plate with the original incoming sample and link this through to the results of the PCR (quantitative or end point). The system will allow retrieval of data by any parameter and will generate an export listing to accompany samples sent to another laboratory. It will also be able to take a list of samples and use the latitude and longitude values to generate a KML file for viewing in Google Earth®, providing an instant overview of the distribution of samples (**Figure 19**).

Figure 17. Data entry screen showing the data fields. Files (e.g. a scan of the original data label) may be linked to an entry.

Figure 18. Individual tubes, storage boxes and freezer shelves are bar coded with a two-dimensional code to make sample indentification and location simple.
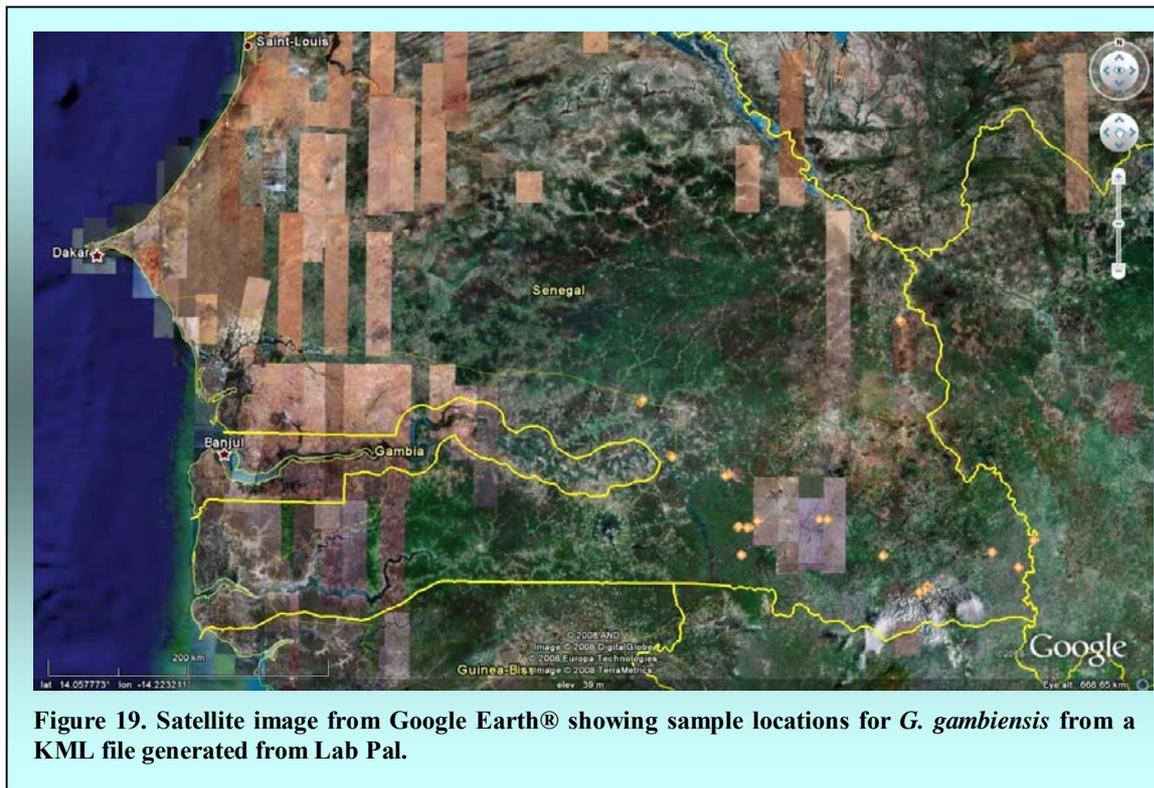
Figure 19. Satellite image from Google Earth® showing sample locations for *G. gambiensis* from a KML file generated from Lab Pal.

3.1.5. Colony Status
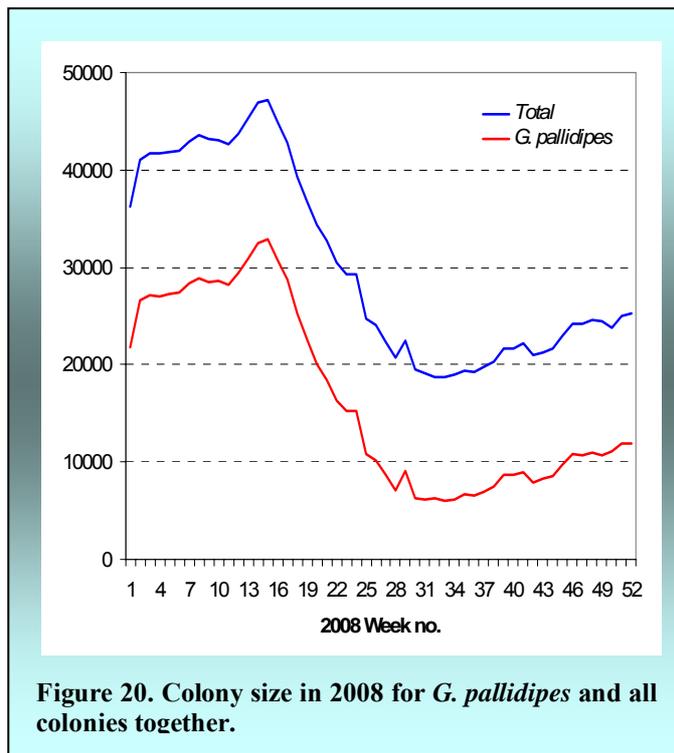
Figure 20. Colony size in 2008 for *G. pallidipes* and all colonies together.
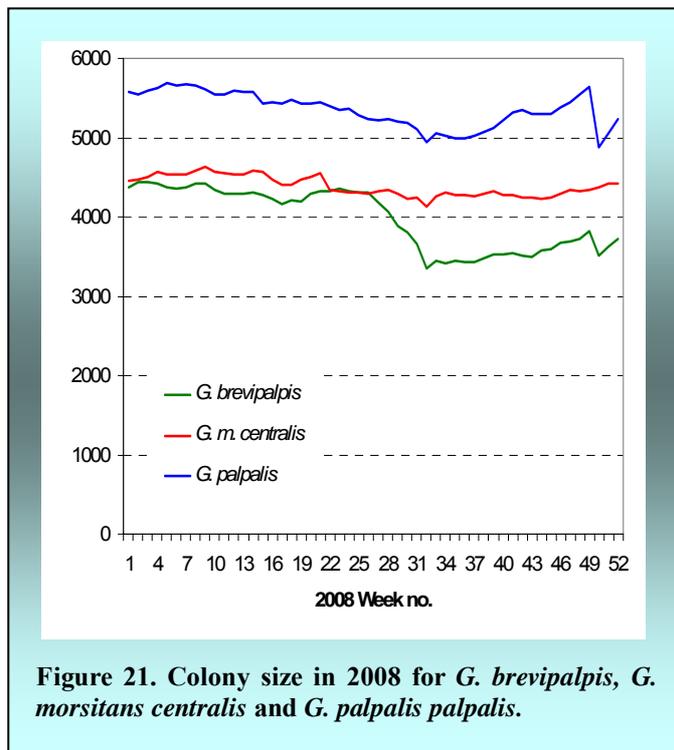
Figure 21. Colony size in 2008 for *G. brevipalpis, G. morsitans centralis* and *G. palpalis palpalis*.

Apart from the *G. pallidipes* colony, the other colonies in the Entomology Unit have performed well throughout the year and have been kept at an approximately constant level (**Figures 20 and 21**). The smallest of the colonies, *G. swynnertoni*, after many years of adaptation has finally started to grow and expanded steadily throughout the year, reaching almost 1700 females.

The *G. pallidipes* colony started the year performing well. Due to a shortage of technicians in the tsetse group, the workload in the Unit needed to be reduced and flies were progressively transferred from standard rearing to the semi-automated feeding and holding system TPU3.2. Input to the standard rearing was stopped in week 15, although the flies already on the standard rearing were kept there and not transferred and continued to produce pupae. This coincided exactly with the peak holding on TPU3.2 of 17 162 females (**Figure 22**). The colony on the TPU3.2 experienced a peak in mortality in weeks 9 and 10, and then rising mortality from week 16 onwards causing the colony to decline rapidly. By week 28, the colony was transferred back to the standard rearing, but mortality remained high for a few weeks. When there is a heavy mortality older flies are preferentially removed resulting in a reduced average age of the colony and increased fecundity. These increased fecundity levels were maintained for the rest of the year even as the colony matured, well above the average fecundity typical in previous years. By the end of the year the colony had recovered to 11 856 females.

The cause of the decline in the *G. pallidipes* colony is not known with certainty. Two main, inter-related factors seem to be involved: infection with the salivary gland hypertrophy virus (SGHV) and maintenance of the colony on the TPU3.2. As reported elsewhere, SGHV

19

Figure 22. Colony size and pupal production in 2008 for *G. pallidipes* on the TPU3.2.

infection causes sterility in those flies that develop pathological signs but flies without visible pathology apparently remain unaffected. It is not yet know which factors lead to the expression of pathology in infected flies, but it is believed that stress plays a role in this.

The development of the TPU3 is still in progress and as part of this development work the transfer of *G. pallidipes* to the TPU3.2 was done, as a result of which we have identified a number of improvements that are needed. Undoubtedly the identified shortcomings in the TPU3.2 will have caused additional stress to the flies (e.g. irregular feeding raised temperature and lowered humidity during feeding) and a sharp increase in the rate of hypertrophy was observed in the middle of the year but this was not accompanied by a marked drop in fertility, but rather by an increase in mortality. Returning the flies to the traditional system quickly reversed the high mortality and, as already noted, also lead to very high fecundity.

3.2. Fruit Fly Rearing and Quality Control

3.2.1. *Effects of Methoprene and Protein on Lekking Behaviour of Male Melon Flies*

Mr Ihsan ul Haq, a doctoral student from Pakistan at the University of Natural Resources and Applied Life Sciences (BOKU), Vienna and a Fellow with the FAO/IAEA has been studying the effects of the juvenile hormone analogue, methoprene, and dietary protein on the lekking behaviour of laboratory-reared male melon fly *Bactrocera cucurbitae* for the purpose of improving the quality of sterile males when used in sterile insect technique (SIT) programmes.

One of the pre-requisites for the success of SIT is that laboratory mass-rearing and sterilization should not adversely affect the mating behaviour of released sterile males. For fruit flies, these males should still be capable of initiating and participating in normal lekking activities and in showing typical antagonistic male-to-male behaviour. Like many other Tephritids male *B. cucurbitae* are reported to engage in lek formation in the late afternoon as precursory activities leading to mating with females that have been attracted to the lek site.

Previous studies on mass-reared *B. cucurbitae* have shown that the addition of protein to the adult diet improved the sexual success of sterile *B. cucurbitae* when compared to released sterile flies fed only on the typical diet of sugar and water. Also it was shown that the exposure of adult males to methoprene accelerated sexual maturity so that they became sexually mature significantly sooner than without methoprene. This method would improve the performance of released sterile males in that they will not have to survive in the field as long as previously before they become capable of mating with wild female flies. Despite these improvements – greater sexual activity and more rapid sexual maturity – the question of whether or not these enhanced flies were actively participating in natural lekking activities remained. The objective of this study was to test the lekking behaviour of male flies whose maturity was enhanced (i.e. protein-fed, and exposed to methoprene) against normally matured males in field cages.

The experiment consisted of four treatments of adult males:

1. M+P+: Adult males exposed to methoprene and fed protein

2. M-P+: Adult males not exposed to methoprene and fed protein

3. M+P-: Adult males exposed to methoprene but not fed protein

4. M-P-: Adult males not exposed to methoprene and not fed protein

Males of the M+P+, M-P+ and M+P- treatments were six days old and those for the M-P- were 14 days old. This was done to take account of the fact that adult males in the absence of methoprene and dietary protein reached sexual maturity some six to eight days after those treated with one or the other or both. All males (20 from each treatment) were marked on the thorax by different-coloured water-based paint one day prior to the commencement of the experiment and released together 90 min before sunset on a non-fruiting citrus tree in a field cage. Lekking behaviour was observed in the absence of females. Then, some 45 min later, 20 females were released and lekking behaviour was again studied until the males had completed sexual activity. By this time complete darkness had set in.

Figure 23 Correlation between *Bactrocera cucurbitae* male dominance and mating success.
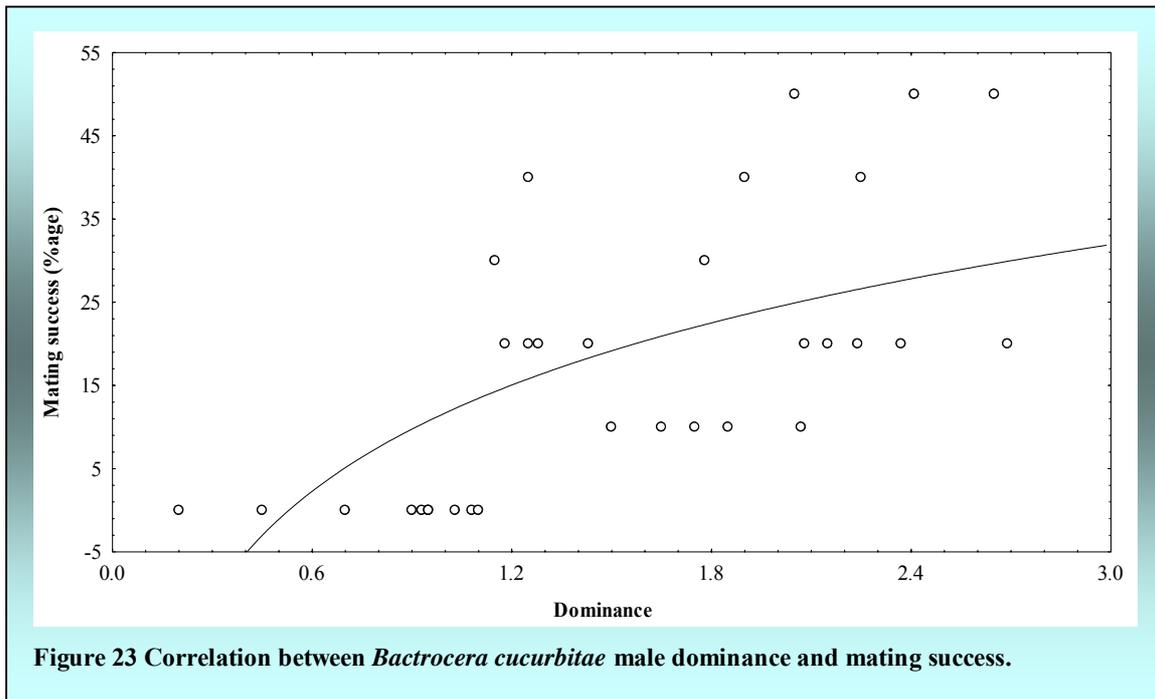
Young (six day old) males treated with methoprene and protein (M+P+) initiated sexual calling significantly earlier than six day old males fed only protein (M-P+), six day old males treated only with methoprene (M+P-) and the 14 day old control males (M-P-). Lek initiation and lek participation were significantly higher by M+P+ males than any other type of male (M-P+, M+P- and M-P-). The combination of methoprene and protein improved male dominance in lekking and was positively correlated to mating success (**Figure 23**). It appeared that the addition of protein to the pre-release adult diet synergised the beneficial effect of methoprene in terms of a higher mating success rate but methoprene alone did not improve the lekking behaviour or mating success (**Figure 24**).

Figure 24. Mating success by male *Bactrocera cucurbitae* treated with methoprene and/or protein.
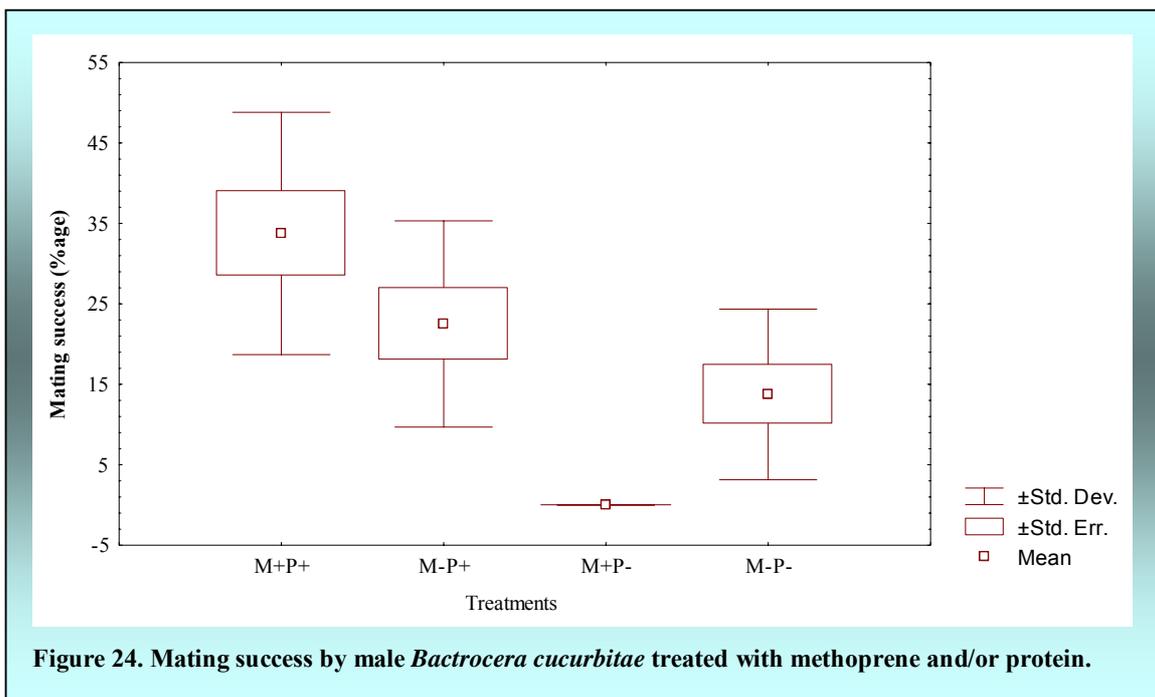
3.2.2. Preliminary Data on Improving Mass Rearing of the Olive Fruit Fly

The olive fruit fly *Bactrocera oleae* is a serious pest or potential pest of olives wherever they are grown around the world. As such control programmes have been implemented in countries where the pest is native or has become established and border protection and monitoring procedures have been set up in olive producing countries where olive fruit fly has not yet invaded. The olive fruit fly exists in most Mediterranean countries where olives are grown and, depending on the season, can inflict massive (up to 90%) losses annually. This results from direct effects on the fruit for both table olive and oil production and in insect-mediated fruit drop in the orchard. Additionally, if insecticides need to be used to control this pest there is a subsequent decrease in product value, problems with insecticide residues in olive oil, and possible damage inflicted on the environment and human health.

If suppression of the olive fruit fly with the incorporation of the sterile insect technique into olive fly management programmes is to be achieved, a simple, efficient and cost-effective mass-rearing system must be developed. The following components of laboratory rearing of olive fly, which are part of the rearing system in use at Seibersdorf, and which are similar to those used in other olive fly research facilities, have been targeted for improvement: (a) *Egg collection*: replacement of egg collection cones with cage-length egg collection panels; (b) *Egg handling*: shortening the incubation period of eggs from 48h to 0h (i.e. no incubation period prior to diet seeding), and (c) *Larval diet*: partial, or total, replacement of cellulose fibres with corn cob grits.

3.2.2.1. Egg collection

The most common method used to collect eggs from laboratory cultured olive flies is by suspending, pointed end down, several (up to six, depending on cage size and shape) "cones" constructed of Ceresin-coated finely woven nylon cloth through circular ports through the ceiling of the cage; a strip of damp sponge is placed in the centre of each cone to reduce egg desiccation.

Many artificial oviposition substrates for laboratory reared olive fly have been tested as alternatives to the cones. Over the last two years, in an effort to improve mass-rearing techniques by increasing egg production and egg quality whilst ensuring a practical methodology for mass rearing, the Unit replaced the cones with plastic bottles with panels cut out from the bottle sides and replaced with panels of the same wax-coated cloth used to construct the cones. The change from cones to bottles as oviposition devices reduced contamination problems, caused by the presence of the damp sponge near the eggs, and also made it easier to place and replace egging devices during the life of the cage. However it was seen that even with these modifications and improvements the production of the bottles for egg collection and the difficulty in placing them inside adult cages and removing them

Figure 25. A flat panel cage for rearing olive fly.

still remained.

This led to further research on improved egg collection methods and cages of different sizes were tested where flat panels of wax-coated cloth were used as egging devices, instead of cones or plastic bottles, in order to increase egg production and egg viability whilst improving the ease of set up and egg collection (**Figure 25**). Several cage designs have been, or are in the process of being, tested for optimal egg output per female. These cages were named according to the surface area of the egging panel that females inside the cage are exposed to. The cages that were tested were as follows:

1. Small (one egging panel at 375 cm^2),

2. Medium A (one egging panel at 936 cm^2),

3. Medium B (two egging panels at 936 cm^2 each giving a total area of 1872 cm^2),

4. Flat (2806 cm^2)

5. Large (4704 cm^2).

Different densities of flies were tested; the eggs were collected during 15 days and the number of eggs produced per female per day was estimated. As a reference for determining the number of eggs collected and the quantity of pupae placed in the cages it was necessary to determine the number of eggs per mL and the number of pupae per mL. We found an average of 34 344 eggs/mL and 79 pupae/mL following standardized procedures which we used in all cases for olive fly studies. The number of females per cage was calculated from the quantity of pupae placed into the cage, the adult eclosion rate for that cohort of flies and the sex ratio determined from the adult eclosion test. The results showed that it was possible to collect eggs from a flat surface and the number of eggs laid per female per day was related to the

Figure 26. Egg production per female per day for olive fruit fly in different types of cages.
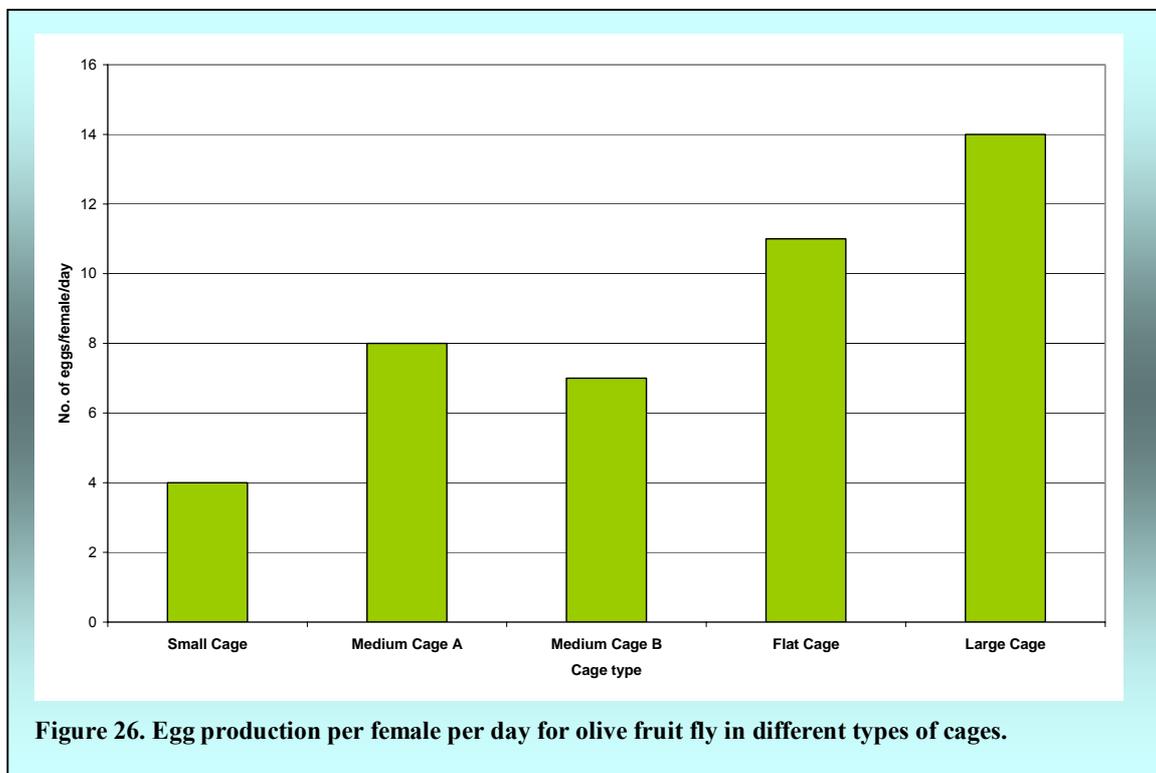
surface area of the egging panel to which females were exposed (**Figure 26**). However more research is needed to determine the optimal density of flies per cage based on the surface area and pupal size.

3.2.2.2. Egg handling

Once eggs are collected, by whatever egging device, the transfer of eggs to the larval rearing diet is the next critical step. At the Unit, eggs were incubated on white filter paper in Petri dishes soaked with propionic acid solution (0.3%). These Petri dishes, with filter paper and eggs were then placed in a sealed plastic container (1L) containing 2 mm water to maintain high humidity around the eggs to prevent desiccation. Incubation time for the eggs at 25°C was reduced from 72h to 24h. It appears that these egg incubation times have been copied from methodologies used for mass-rearing other fruit fly species, notably Mediterranean fruit fly *Ceratitis capitata*.

Using the standard methodology for incubation a test was carried out with different periods of incubation: 0h, 24h, 48h and 72h after egg collection. Previous research at the Unit had shown that the exposure of eggs to proprionic acid was detrimental to egg hatch and subsequent maturation of neonate larvae to pupation and adult eclosion (unpublished data). Batches of 100g of standard olive fly larval diet, based on cellulose fibre, were seeded with eggs from each incubation period (treatment). After 14 days under standard olive fly larval rearing conditions, surviving larvae were separated from the medium and allowed to pupate in sawdust. Percentage egg to pupa recovery was calculated from the number of pupae collected compared with the number of eggs seeded onto the larval diet. The goal of this experiment was to increase egg to pupa recovery. The results show a clear correlation between egg to pupa recovery and reduced incubation time. Based on this result, egg incubation has been eliminated from our laboratory protocol, and optimal egg density was established at 10 eggs/gram of diet.

3.2.2.3. Larval diet

The larval diet used at Seibersdorf is comprised of the following components. For 1 kg of larval diet, tap water (550 mL), extra virgin olive oil (20 mL), Tween 80 Emulsifier (7.5 mL), potassium sorbate (0.5 g), Nipagin (2 g), sugar (20 g), brewer's yeast (75 g), soy hydrolysate (30 g), hydrochloric acid 2N (30 mL) and cellulose fibre (275 g).

Cellulose fibre (or powder) has been identified as one of the more expensive consumables used in the artificial rearing of olive fly. Cellulose fibre is an inert bulking agent. It has been incorporated into the olive fly diet to improve the diet's physical characteristics by improving the insect's ability to move through the diet to access food, water and oxygen. It is used as the diet bulking agent in most facilities that rear laboratory cultures of olive fly.

Experiments were set up to test a low-cost larval diet using corn cob grits to completely or partially replace the cellulose fibre. Here 300 g batches of larval diet were made up using the standard Seibersdorf ingredients and procedure. These were labelled as the controls. Test batches of larval diet were made up, again according to standard practice, but with the standard bulking agent (cellulose fibre) replaced with the same weight of corn cob flour/grit (Mt. Pulaski Products Inc. Mount Pulaski, Illinois, USA), or with the same weight of a mixture of corn cob flour/grit with cellulose fibre.

The treatments were:

1. 100% cellulose fibre (the control)

2. 50% by weight cellulose fibre and 50% of corn cob flour/grit

3. 100% corn cob flour/grit

Three corn cob flour/grit products were tested:

1. Cob flour #100: US Standard Mesh - 55

2. Cob flour #4: US Standard Mesh - 30/80

3. Cob grit #3: US Standard Mesh - 20/40

A test was carried out to evaluate the efficiency of these diets. Eggs from the same strain and collection day were seeded onto the test diets (0.05 mL of eggs in 100 g of diet per replicate). To determine larva to pupa recovery, egg hatch was determined from 3 x 100 egg samples placed on black filter paper in Petri dishes. All pupae recovered were weighed on the 8th day after pupation to ensure consistency. The number of pupae collected from each diet was recorded. Adult eclosion rates from surviving pupae were calculated by placing all recovered pupae in labelled Petri dishes at 25°C. The number of adult flies normally eclosed (i.e. fully emerged with wings and body fully expanded) for each treatment was recorded. Five replicates were carried out over time using different cohorts of olive fly. New batches of larval diet were made for each replicate. Environmental conditions were a constant temperature of 25ºC and 60% RH. Egg to pupa recovery rates, for each diet treatment, are shown in **Figure 27**. The results suggest that diet with 50% cellulose-50% corn 1 (i.e. Cob flour #100: US Standard Mesh -55) can be used instead of 100% cellulose. However further research is needed to evaluate diet development (humidity, texture, fungal and other micro

Figure 27. No. of olive fly pupae recovered from different diets based on cellulose and corn cob flour/grits alone and in combination (5 replicates of approx. 1500 eggs in 100g of diet).
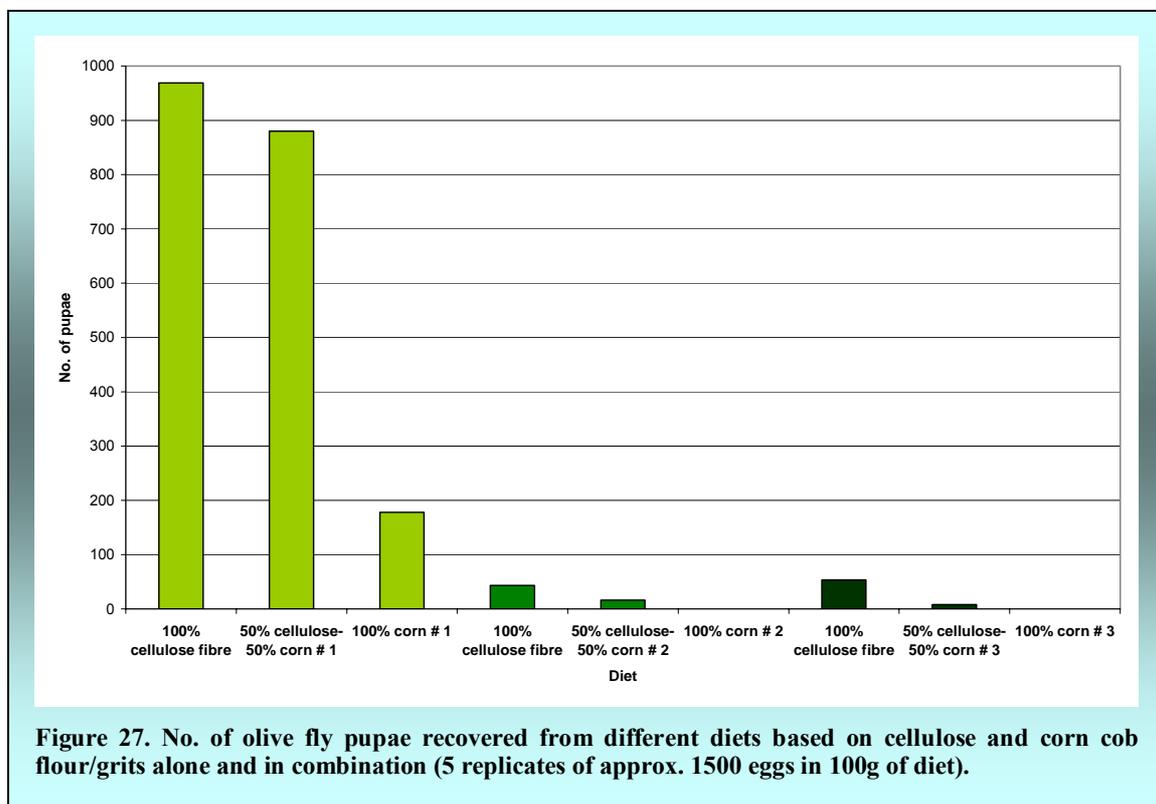
flora contamination), quality of the flies and egg pupae recovery through several generations under laboratory conditions.

Results on the egg collection system, as well as larval and adult diets require more replicates and fine tuning. The current weakness of olive fruit fly mass rearing is handling of mature larvae and larvae separation from the diet. Further research will focus on this process.

3.2.3. *Importance of Egg Yolk in the Adult Diet of Laboratory-Reared Olive Fruit Fly*

In most small-scale facilities laboratory-reared adult olive flies are routinely fed a mixture of chicken egg yolk, hydrolysed protein, sugar and water. This is an unusual requirement as no other laboratory-reared fruit flies are fed chicken egg yolk and it is unlikely that adults feed on this substance in nature. Chicken egg yolk as an addition to the olive fly adult diet was first studied by Aris Economopoulos in the 1970s who found that its addition resulted in an improvement on the volume of eggs produced from caged olive flies. Since then most laboratories that rear olive fly use chicken egg yolk in various forms in adult diets.

The hypothesis presented by Economopoulos was that the yolk of a chicken egg improved egg production from olive flies that fed on it by adding fatty acids and lipids to their diet. Optimal adult nutrition is an important aspect for rearing of olive flies (and all other species used for SIT) and if this insect is to become a good candidate for SIT then it is essential to know what diet ingredients will ensure adequate performance of the sterile insects in the field.

The present studies used a laboratory culture of olive fly developed from a ten generation-old cross between a very old laboratory culture called the "Demokritos" strain (on which are based many olive fly laboratory strains around the world) with an Israeli strain which had been reared in the laboratory for ten generations after collection from the wild. Flies were housed under 25°C, 65% RH and 14h light : 10h dark) conditions in small cages (66 mm long x 45 mm wide x 45 mm high) fitted with a feed container and water soaked sponge. Food and water were supplied continuously for the life of the experiment. An egging panel made of fine terylene cloth (15 strands x 15 strands/cm^2) coated with a thin layer of a mixture of waxes was also included in each small cage. The wax mixture was of paraffin (300 g), bees' wax (21 g) and liquid paraffin (18 mL). It was heated to become a soft liquid and the cloth was dipped very slowly into the mixture (to ensure a very thin layer of wax adhered to the cloth) and then allowed to harden at ambient. Either ten males and ten females or five males and five females were housed in each cage.

Four treatments were tested and six replicate cages were set up for each treatment, three of which had the panel removed each day for egg collection and replaced with a fresh one and the other three of which had the same egging panel remain in place for the length of the experiment. The four treatments were:

1. **FEY+PH+S+W:** 5 g of adult diet made of fresh egg yolk (30 g) (made by collecting egg yolk from fresh eggs, beating them and spreading the yolk in a thin layer to be dried in an incubator at 60°C for three days, then pulverised) plus protein hydrolysate enzymatic (100 g) (MP Biomedicals, Germany) plus white crystalline sugar (400 g). Water was supplied *ad libitum*. Cages used for this treatment housed ten pairs of flies.

2. **IEY+PH+S+W:** Adult diet of industrial egg yolk (Inovo, Germany) plus one part protein hydrolysate to three parts white crystalline sugar. Water was supplied *ad libitum*. Ten pairs of flies per cage.

3. **PH+S+W:** Adult diet of one part protein hydrolysate to three parts white crystalline sugar. Water was supplied *ad libitum*. Ten pairs of flies per cage.

4. **S+W:** Adult diet of sugar only. Water was supplied *ad libitum*. Five pairs of flies per cage. During the experimental setup stage for this experiment we decided to use this number of flies as we considered that, based on our experience with other fruit fly species, flies fed only sugar and water would be unlikely to produce fertile eggs.

The PH+S+W feeding regime is the standard feed and water used for all fruit fly rearing at Seibersdorf. Cages were opened for egg collection at 10:00 am every 24h for eight days from the time the adults commenced oviposition. Eggs were then washed free from the egging panel and the cage reclosed. Eggs were counted and the number was recorded. Also the number of dead males and females in each cage at the time of egg collection was counted and recorded. Results showed that there was no difference in egg production whether the egg panel was replaced each day with a fresh panel or not. Data from the two sub-treatments were pooled (i.e. six replicate cages per treatment).

The experiments showed that the fresh egg yolk additive gave results, with respect to egg production and both male and female longevities tested in this experiment, which were superior to the three other treatments. There were significant differences, at the 5% level of significance, in both the total quantity of eggs produced and the total quantity of these eggs that hatched between treatments. The standard diet with the addition of egg yolk prepared from fresh eggs was superior to the standard diet which was, in turn, more productive than both the standard diet with industrial egg yolk and the sugar-only diet (there was no significant difference between these latter two diets). Percentage egg hatch, however, did not vary significantly with adult diet treatment. The main factor in the differences between adult diet treatment effects was due to treatment-induced adult mortality (**Figure 28**). Both male and female survival declined with age over the eight days of the experiment. Female survival

Figure 28. Survival of female olive fruit fly over time on different diets.
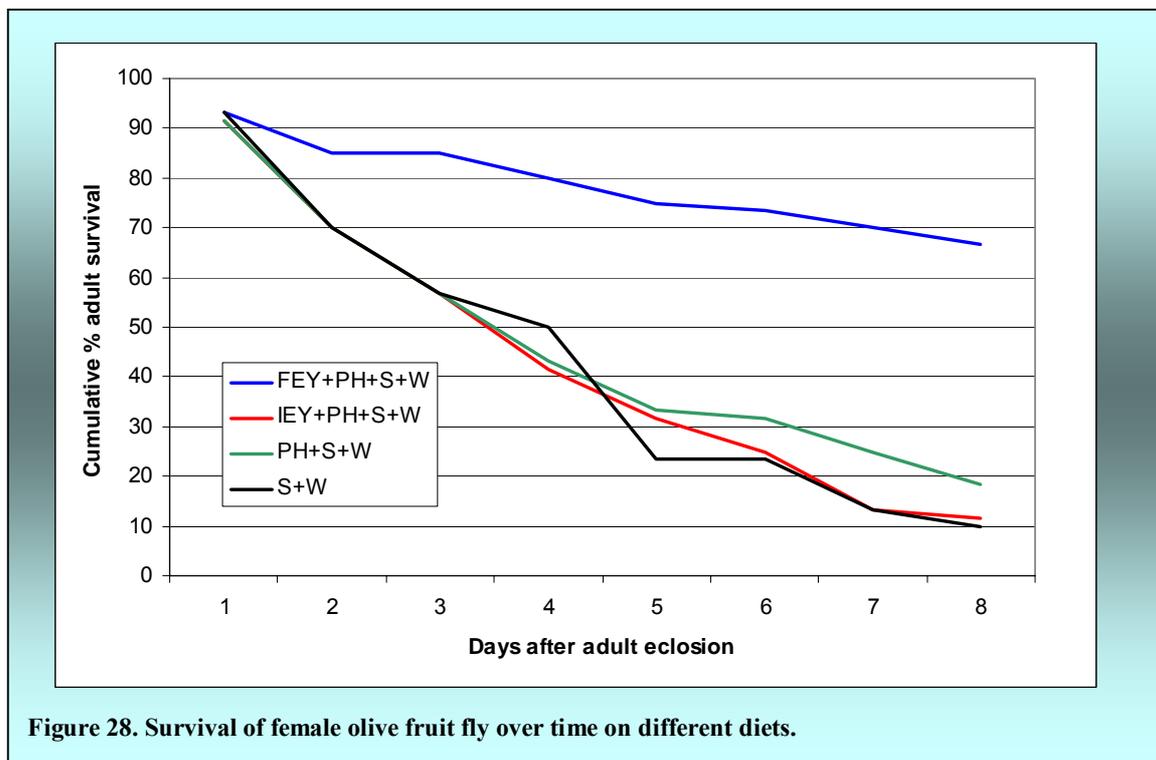
was greatest if fed the fresh egg yolk supplemented diet whereas female survival was least whether they were fed the standard, industrial egg yolk enriched standard or the sugar only diets.

The conclusion of this experiment is that the standard protein hydrolysate plus sugar diet, when enriched with egg yolk prepared from fresh eggs, is superior in comparison to the others tested on the basis of fertile egg production. It is unclear if the reason for this improvement is due to the egg yolk being rich in lipids, fatty acids and antioxidants or whether the protein/amino acid mix in the egg yolk is more beneficial than that given in the standard protein hydrolysate. Another factor to be considered is the texture of the adult feed. There may be differences in the accessibility of adult flies to nutrients due to variation in the ease of feeding on each of the treatment diets used in this experiment. Future work on this should try to ensure that each diet is of a similar consistency. Also the adverse impact of extra protein on adult survival due to enhanced sexual activity (i.e. increased energy expenditure due to an increased rate of reproduction) should be considered. There may be other reasons for this observation given that the addition of industrial egg yolk was, in most ways, inferior to the standard protein hydrolysate/sugar mix. A future experiment will test flies fed on fresh egg yolk plus sugar (but no protein hydrolysate) and fresh egg yolk plus protein hydrolysate but no sugar.

3.2.4. *Interspecific Crosses between the Oriental Fruit Fly and the Invasive Fruit Fly*

Invasive pest fruit flies (Diptera: Tephritidae) are of major economic importance in nearly all tropical and subtropical and many temperate countries world wide. Several representatives are known to attack different types of commercial and wild fruit and vegetables, causing considerable damage to agricultural crops. Some formally minor species have increased significantly in importance. Most regions of the world implement border control procedures at gateways into those regions, such as quarantine control at airports and shipping terminals. They also set up national fruit fly trapping grids to intercept incursions of exotic pest species. Despite these precautions there have been instances of incursions of exotic pest fruit flies for example the Mediterranean fruit fly *Ceratitis capitata* has now spread almost worldwide, the Oriental fruit fly *Bactrocera dorsalis* and the melon fly *B. cucurbitae* in Hawaii, the olive fly *B. oleae* into California and Mexico and the invasive fruit fly *B. invadens* into the African continent.

One of the more important aspects of successful border control against the incursion of invasive species is accurate species identification. Only once this is established can the potential new host region implement targeted management, suppression or eradication procedures. Inaccurate species identification may have serious consequences with respect to restriction of trade from that region to other regions that are free of this pest and also the likelihood of wasting finances on pest mitigation schemes not suited to the new species. This issue arises in the case of distinguishing between Oriental fruit fly *B. dorsalis* and the invasive fruit fly *B. invadens*.

Correct species identification is a pre-requisite for the effective use of the sterile insect technique. Additionally there have been instances where insects of the same species behave differently depending on geographical location (e.g. oranges are host to the South American fruit fly *Anastrepha fraterculus* in Brazil but not in parts of Northern Mexico) leading some taxonomists to suggest that speciation has occurred. Effective SIT programmes need to be

aware of this possibility and research needs to be carried out to ascertain its relevance. In fact it has been suggested that SIT programmes implemented in various regions would be improved by hybridizing laboratory-adapted cultures of the target pest with local populations to take account of slight variations in genetic makeup. Hybrids may combine advantageous traits from both parental species. For example the intraspecific hybridization of a laboratory colony with the local wild population may result in a culture that combines the laboratory adaptation with the ability to survive under local climates and types of food sources.

Interspecific hybridization is generally less sustainable than the parental strains because the offspring from such unions is generally infertile. However there have been instances of successful interspecific hybridization in the laboratory (e.g. the Queensland fruit fly *Bactrocera tryoni* with Jarvis' fruit fly *Bactrocera jarvisi*, and the Oriental fruit fly with the carambola fruit fly *Bactrocera carambolae*). Such hybridization in nature has been postulated between closely related species such as Queensland fruit fly and the lesser Queensland fruit fly *Bactrocera neohumeralis,* and between the Philippines fruit fly *Bactrocera philippinensis* and *Bactrocera occipitalis*.

The verification of the physiological potential for interspecific hybrid production has additional implications. It is possible that two insect populations from the same species have become separate species due to geographical isolation but are still sexually compatible. In this case interspecific hybrids may be useful for SIT programmes across broad geographic ranges.

A series of experiments has been set up in stages to assess the sexual compatibility between *B. dorsalis* and *B. invadens* and the development of a hybrid strain. The initial step is to force interspecific mating in small laboratory cages under laboratory conditions. The second is to offer females of each species a choice between mate species. The third is to carry out mating competitiveness experiments under simulated natural conditions in field cages. If fertile hybrid offspring result from interspecific crossing, quality assessment tests on their offspring will be carried out.

B. dorsalis and *B. invadens* are difficult to distinguish from each other based on morphological and behavioral characteristics. The objective of this study was to assess, first, the possibility of interspecific mating between these two invasive species and, secondly, the quality (based on SIT standards) of any hybrids produced. The potential of using such hybrids for SIT programmes against both *B. dorsalis* and *B. invadens* will then be assessed.

Flies used in the hybridization experiments described here were laboratory cultures of *B. dorsalis* (Hawaiian genetic sexing strain) and *B. invadens* (culture from Kenya reared in the laboratory for 59 generations). For these experiments laboratory cultures of both species were reared on the same diet ("Seibersdorf diet" based on wheat bran) under the same laboratory room conditions (75% RH, 14h light:10h dark photo period and a constant temperature of $24°C ± 1°C$). The same egg density per unit volume of larval diet (3.2 mL eggs/4 kg diet) was used for each species. Virgin flies used in the experiment were segregated by sex within two days after adult eclosion. All fly transfers during the experiment were done with gentle aspiration. All adult flies were the same age and kept under the same environmental conditions (60% RH, temperature at 24°C and a photoperiod of 14h light: 10h dark). Flies were kept in a small plexi glass cage (17 cm long x 7 cm wide x 12 cm high) with food (1:3 yeast hydrolysate:sugar) and water. Five flies of each sex were housed in each cage. This

experiment was replicated three times (i.e. three replicate cages per crossing) but all flies within each species were from the same cohort. There were four treatments:

1. Bi x Bi: *B. invadens* male crossed with *B. invadens* female

2. Bd x Bd: *B. dorsalis* male crossed with *B. dorsalis* female

3. Bi x Bd: *B. invadens* male crossed with *B. dorsalis* female

4. Bd x Bi: *B. dorsalis* male crossed with *B. invadens* female

Small plastic vials with a few drops of guava juice were used as artificial oviposition devices (for egg collection). These "egg cups" were placed in each cage for 24h five times over the subsequent three weeks from the day of first oviposition (day 11 after adult eclosion under the environmental conditions described above). Collected eggs were incubated for 24h on damp filter paper in Petri dishes and then transferred onto the "Seibersdorf starter diet", which is based on dehydrated carrot, and incubated for 96 hours (85% RH and 25°C). The starter diet was used as it was anticipated that eggs from the hybrid crosses may be difficult to hatch. Mature larvae were allowed to exit the larval diet and pupate in saw dust.

The quantity of eggs produced during each 24h egg collection was recorded and, after incubation on damp filter as described above, we assessed the quantity of those eggs that hatched (i.e. were fertilised). Eggs from the interspecific crosses (but not those from the parental matings) were transferred to larval diet and the percentage egg to pupa recovery was calculated.

The first trial showed that the two species mated with each other under forced laboratory conditions. Eggs were produced as a result from both interspecific crosses (male *B. invadens* crossed with female *B. dorsalis* and the opposite cross). These eggs were viable and produced

Figure 29. Egg production from intra- and inter-specific crosses between *Bactrocera dorsalis* and *B. invadens* (Total eggs produced from 5 fly pairs / treatment from 5 egg collection days; average from 3 replicates).
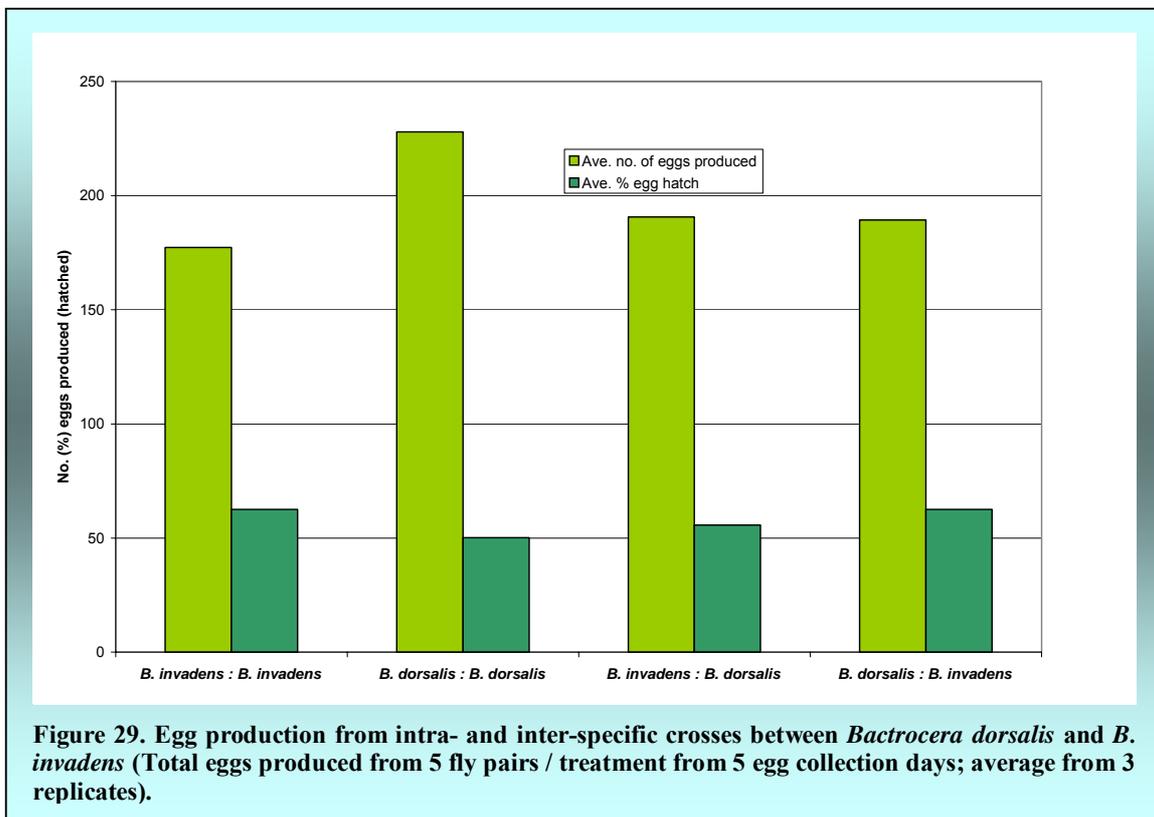
adult F_1 hybrids. There were no significant differences in the quantity of eggs produced between the two specific crosses and the two interspecific crosses at the 5% level of significance. Egg production by the Bi x Bi cross tended to have fewer eggs than the Bd x Bd cross. The quantity of eggs produced from the two interspecific crosses was intermediate between the Bi x Bi cross and the Bd x Bd cross. There were no significant differences in percentage egg hatch between the four crosses (**Figure 29**). The first egg collection from crosses with any *B. invadens* parent (i.e. Bi x Bi, Bi x Bd and Bd x Bi) produced low egg quantities with low egg hatch rates. This could be due to the slightly lower than optimal maintenance temperature used for *B. invadens* in these experiments. In Kenya laboratory flies are kept at 28°C rather that the 24°C used in the experiments reported here and may be slightly less mature than the *B. dorsalis* adults. The percentage recovery of pupae from hatched eggs for both interspecific crosses was remarkably high especially in the latter egg collections when adults had matured (**Figure 30**). The next phase of the experiment will be to repeat the above experiment, once with the Hawaiian genetic sexing strain and then with a normal non-sexing strain reared from wild fruit collected in Thailand. Following that, trials of non-forced, choice matings in the laboratory and then in field cages will be carried out.

3.2.5. Comparison of the Effects of Gamma Radiation and Low-energy X-rays on the Sterility and Quality of Fruit Flies (Diptera: Tephritidae) for Sterile Insect Release

During 2008 three fellows, Raza Memon from Pakistan, Jaime Palma from Chile and Thiago Mastrangelo from Brazil, conducted a series of experiments comparing two different technologies that can be used to sterilize insects for the sterile insect technique. Both technologies produce ionizing energy to which fruit fly pupae are exposed to induce sterility but the energy is produced by two different sources. The more commonly used technology produces ionizing energy from gamma radiation emitted by the radioisotope ^{60}Co and the

Figure 30. Percentage egg to pupa recovery over time from adult eclosion for F_1 offspring from reciprocal crosses between *B. invadens* and *B. dorsalis*.
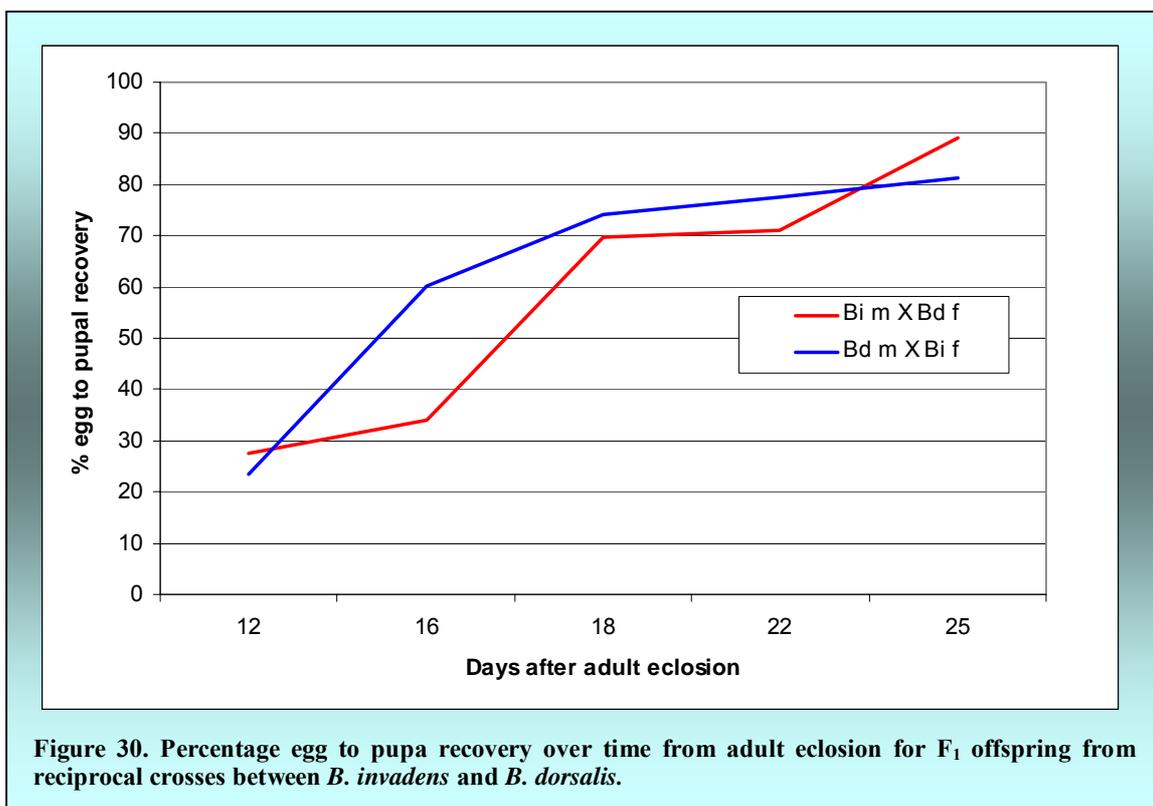
second produces electrically generated ionizing energy from X-rays produced by a self-contained low-energy irradiator.

In recent years, it has become increasingly difficult to set up new SIT programmes around the world where ^{60}Co and the alternative radioisotope ^{137}Cs are to be used to sterilize insects. The shipping of such radioisotopes has become extremely difficult due to nations concerns for their nuclear safety in case of accidents and theft. Also some regions may not have radiation safeguards in place for the application of this technique or procedures in place in case of accidents. An electrically generated radiation source such as is provided by low-energy X-ray technology may, if suitable, be a practical alternative to ^{60}Co and ^{137}Cs allowing SIT programmes to be set up on a more global scale.

The aim of this work was to ascertain if there are any differences between gamma rays and X-rays in adult sterility and those quality parameters that impact on the success or otherwise of SIT programmes. If it can be demonstrated that there is no difference in the sterility and quality indices of adult flies whether they are sterilized by gamma radiation or by X-rays then the two technologies could be used interchangeably. Practical factors such as cost, regional approvals, supply and demand for the technology, use for other applications (e.g. quarantine treatment) and others will also play a role in the degree to which either technology is utilized.

Experiments tested the two technologies on pupae of the Mediterranean fruit fly *C. capitata*, the South American fruit fly *A. fraterculus*, and initial experiments on the melon fly *B. cucurbitae*. These three species were considered to be a sound representative sample of species, origin and pest status.

The experiments took place in controlled environment rooms (23±1ºC, 65% RH and a photoperiod of 14h:10h light:dark). The *C. capitata* strain used was the *tsl*-VIENNA 8/D53, for *A. fraterculus* a wild type strain was used established from an Argentinean population in 2006 and for *B. cucurbitae* a wild type strain was used that was established from pupae received from the Seychelles in early 2008.

Pupae were reared following normal procedures in the Seibersdorf laboratory and were exposed to gamma radiation via a ^{60}Co irradiator (Gammacell-220), or to X-rays generated in a self-contained low-energy irradiator, the prototype RS-2400 (Rad Source Inc., Georgia, USA). In the Gammacell test pupae were placed in small plastic bags (approximately 350 g) which were sealed with heat and then placed in the Gammacell for the required period to receive the target dose. In the X-ray irradiator pupae were placed in a 375 mL plastic tube in the centre of a metal canister (178 mm diameter and 167 mm in length) filled with grains of rice. The canisters (5 of them) were positioned in the machine so that they revolved around a horizontally-positioned X-ray tube. For each batch of pupae, dosimetry was confirmed with the Gafchromic® dosimetry system.

Treated (either by gamma radiation or X-rays) and control (not treated) pupae, were either set aside under controlled laboratory conditions until adult eclosion or set up for assessment of percentage adult eclosion, adult flight ability and adult longevity under stress. Those set aside until adult eclosion were then separated by sex prior to sexual maturity and set up with fertile partners for assessment on fertility and fecundity and mating competitiveness in field cages. All assessments followed standard FAO/IAEA/USDA Quality Control assessment procedures.

To date the full range of experiments as described above on *C. capitata* and *A. fraterculus* have been completed and work on *B. cucurbitae* is underway. For *C. capitata* and *A. fraterculus* no significant differences have been found between the two types of irradiation sterilization in any of the quality control tests performed. Preliminary data for *B. cucurbitae* suggest a similar outcome but more replication is required to reach the number of replicates carried out for the former two species.

3.3. Fruit Fly Genetic Sexing

3.3.1. *A Standard Polytene Chromosome Map for the Mexican Fruit Fly*

Polytene chromosomes have proven to be an essential tool for the genetic analysis of *Drosophila* since the first publication in 1935 of the hand-drawn maps of Bridges. Polytene chromosome maps are available for about 270 species of Drosophilids and for more than 250 other Diptera. Genetic and cytogenetic analyses of Diptera have been greatly facilitated by the existence of these maps because of the reproducible banding pattern of polytene chromosomes within each species. Polytene chromosomes are also an excellent material for studies related to the chromosome structure and function, gene activity, phylogenetic relationships among closely related species and are used to distinguish members of a complex species group. Polytene chromosome maps are essential tools to determine the structure of chromosome rearrangements such as translocations and inversions which are used in basic genetic studies as well as in practical applications. They also provide a means for accurate mapping of any cloned DNA sequence by *in situ* hybridization thus contributing to the construction of detailed genetic/cytogenetic maps.

For insect pest species such studies can make a significant contribution both to the understanding of population variations and to the development/improvement of control methods. The development of polytene chromosome maps for *C. capitata* has helped to improve the sterile insect technique by supporting the development of genetic sexing systems.

The Mexican fruit fly *Anastrepha ludens* is a major pest for a variety of fruits in Central America from Mexico to Panama. In addition, its natural distribution includes the Rio Grande Valley of Texas while invasions have been observed in Southern California and Arizona. The species is the target of an intensive suppression programme in Texas and an eradication campaign in Mexico. In spite of its economic importance no genetic or cytogenetic information exists for this species. Only one report, published by Guy Bush in 1962, describes the mitotic karyotypes of nine *Anastrepha* species including *A. ludens*.

The Tephritidae family, to which *A. ludens* belongs, has a diploid chromosome number of 12, including an XX/XY sex chromosome pair. The first cytological data for *A. ludens* were reported by Bush in 1962. He presented a total of six pairs of acrocentric chromosomes, including a pair of heteromorphic sex chromosomes. Our results are in agreement with the above report. The heterogametic karyotype (XY) is usually ascribed to the male. In fact, this is the case for all Tephritidae species analyzed so far, including *C. capitata*, *B. oleae, B. tryoni, B. cucurbitae*; *B. dorsalis* and several species from the genus *Anastrepha*. Male heterogamety in *A. ludens* is supported by the availability of Y-autosome translocations in this species.

Both sex chromosomes seem to be highly heterochromatic based on Giemsa staining and C-banded metaphase chromosomes. These are easily identified because they are the only C-banded chromosomes of the complement and
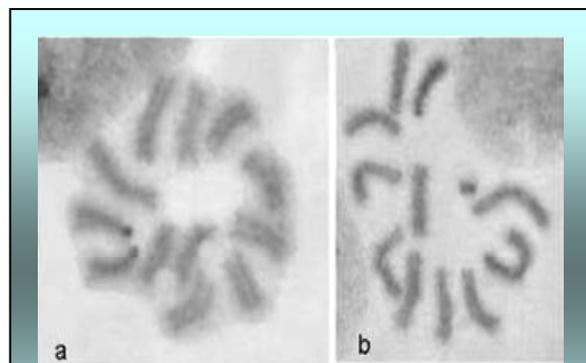

Figure 31. *Anastrepha ludens* **mitotic metaphase chromosomes from larval nervous ganglia: C-banding from female larva (a), and male larva (b).**

they show a different degree of chromatid separation as compared to the autosomes (**Figure 31**). The Y chromosome is the smallest, almost dot-like, chromosome.

In *A. ludens* polytene nuclei no typical chromocenter is present. A chromocenter functions by attracting the centromeres of all the polytene elements to one location. An analogous situation exists in *C. capitata*, *B. oleae*, *B. tryoni* and the sheep blowfly *Lucilia cuprina*. The lack of a chromocenter complicates the exact location of the centromere. Based on several criteria and taking into account the mitotic karyotype and the chromosome morphology during anaphases, the most likely centromere position for each chromosome was ascertained. An additional observation that possibly supports the proposed centromere location is the ectopic pairing observed between the centromeric areas of chromosome II and III. A similar situation, described as a "partial chromocenter", has been found with chromosomes 2 and 3 in *B. tryoni* and is attributed either to the large amount of heterochromatin or to the high level of polytenization at the centromeric regions of these chromosomes. It is not known if this explanation holds true for *A. ludens* but the fact that this specific ectopic pairing between chromosomes II and III was mainly observed in the largest, highly polytenized nuclei seems to support the above explanation. However, all these conclusions need further confirmation by analyzing mitotic and polytene chromosomes from larvae carrying translocations, especially those with breakpoints close to chromosome ends.

Five banded polytene chromosomes were observed in *A. ludens* salivary gland nuclei, corresponding to the five autosomes but no sex chromosomes were found. This observation is comparable with the situation in *C. capitata* where also no banded sex chromosomes were found although a heterochromatic network is present in salivary glands corresponding to the X chromosome and an irregular heterochromatin structure in orbital bristle cells. Banded sex chromosomes were also not found in *B. oleae* and in *B. tryoni*.

The *A. ludens* polytene chromosome reference maps are shown in **Figures 32 to 36.** Chromosomes were labeled from II to VI according to their size; this labeling does not imply any correlation to the numbering of the mitotic chromosomes. As previously mentioned polytene chromosome elements correspond to the five autosomes. The sex chromosomes, which are not polytenized, are labeled as the first chromosome pair. The whole polytene complement was subdivided into 100 sections using the most prominent or distinctive bands as section boundaries. A brief account of the prominent diagnostic landmarks for each chromosome is given below.

Chromosome II, sections 1-22

Chromosome II (**Figure 32**) is the longest chromosome of the polytene complement and has an easily distinguishable banding pattern. The characteristic tip in section 1 and the section 22 proximal to the centromeric region are the most prominent markers for identifying this
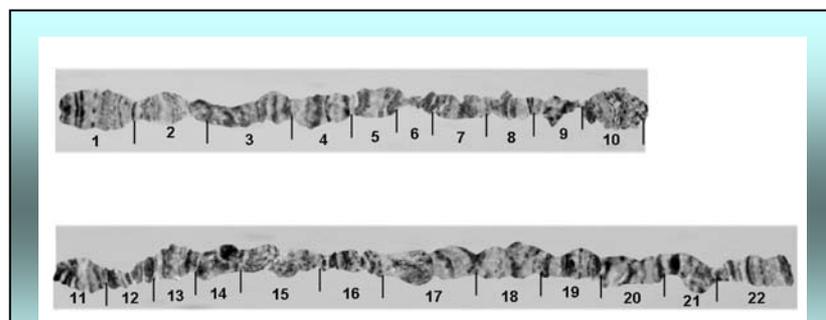

Figure 32. Reference map of chromosome II, sections 1-22.

chromosome. The tip is usually involved in ectopic pairing. Section 5, which is usually followed by a constriction and section 10 that starts with a constriction are regular landmarks. Additional constrictions are observed at the boundaries between sections 10 and 11, in the middle of section 12 and between sections 21 and 22. Puffing is consistently found in sections 17, 18, 20 and 21.

Chromosome III, sections 23-44

Most of this chromosome (sections 25-38) has a very poor banding pattern and numerous weak points at the sections 24, 27, 29, 32, 33, 34 and 38. The tip (section 23) which is frequently involved in ectopic pairing (**Figure 33**) and section 24 are easily identifiable markers for this chromosome. Sections 40-
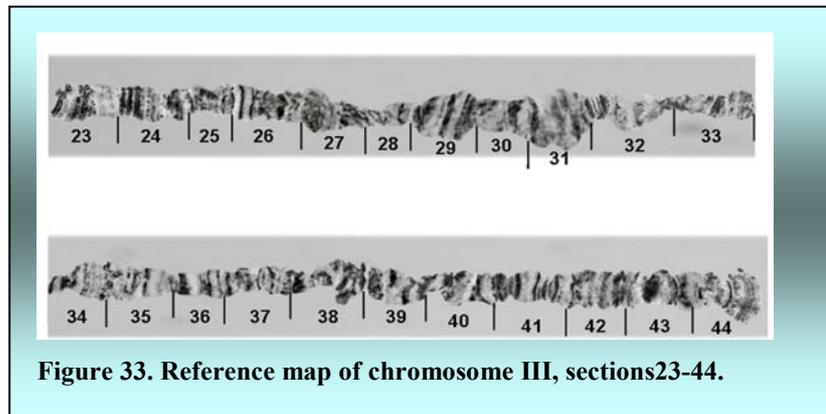
Figure 33. Reference map of chromosome III, sections23-44.

44 have the best banding morphology in this chromosome and sections 41, 43 and 44 are important landmarks for the identification of this chromosome. Section 44 is followed in most cases, but not always, by a diffused heterochromatic area (**Figure 33**) that most likely represents the centromeric area of this chromosome.

hromosome IV, sections 45-65

Chromosome IV is the most distinctive of the polytene complement (**Figure 34**). The tip (section 45) has a characteristic banding pattern and sections 51-52, which are always followed by a constriction, constitute important diagnostic landmarks for this chromosome. Section 56 with its unique pattern is a prominent marker.
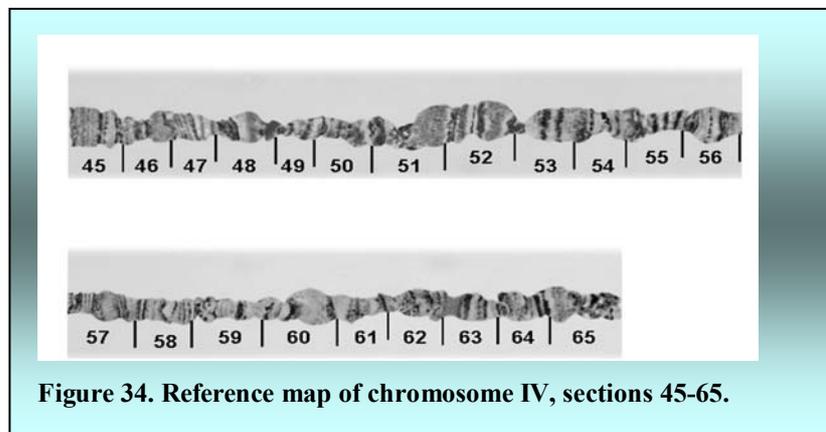
Figure 34. Reference map of chromosome IV, sections 45-65.

Additional diagnostic regions are sections 60, 64 and 65 where the last possibly represents the centromeric region of the chromosome. The diffuse bands at the end of section 65 sometimes have the form of a constriction with a deep dark band.

Chromosome V, sections 66-84

The tip of this chromosome, section 66, is easily identified by its banding pattern. The following sections until the end of 72 have a well-banded morphology in most nuclei. Sections 69, 70, 72 and 76 are regular landmarks for this chromosome. In addition, section 72 is probably a duplication as deduced from the pairing configuration shown in **Figure 35**.

Several constrictions in sections 67, 71 and 73 and weak points at the end of sections 73, 79, 80 and in the middle of section 83 characterize this chromosome element. The most frequent breakpoint is that at section 84, just after the two thin dark bands, which is always followed by a diffuse region. Possibly this area represents the centromeric region of the chromosome.
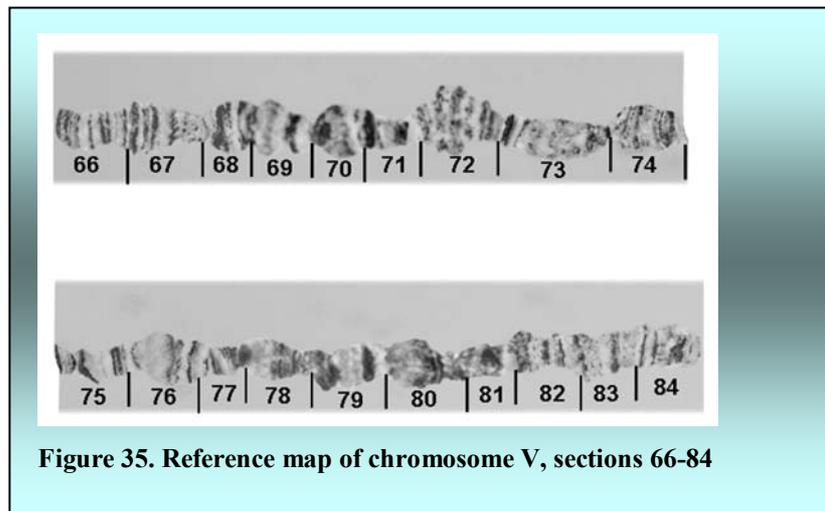
Figure 35. Reference map of chromosome V, sections 66-84

Chromosome VI, sections 85-100

Chromosome VI is the most difficult of the complement due to its poor banding pattern, numerous constrictions and weak points that are frequently broken (**Figure 36**). Important landmarks for the chromosome are the tip at section 85, sections 86 and 91-92. The latter shows a unique banding morphology; the two puffs are always followed by two dark and sharp bands and a light band in the middle (end of section 92). The pairing morphology of section 90 strongly suggests that this region is duplicated. Section 100 has always a weak point and diffuse bands, which might indicate that this is the centromeric region.

An interesting observation is the frequent ectopic pairing between tips of different chromosomes. This has also been observed in *B. oleae*, where this pairing is so complete that it is difficult to identify the free ends of the involved elements. The same phenomenon was also found in *B. cucurbitae* and appears to be widespread among the Tephritidae family. Possibly it is related to the molecular organization of the telomeres in these species. A similar phenomenon has been observed in the *Gaiano* strain of *D. melanogaster* and is attributed to the increased copy number of retrotransposons, *Het-A* and *TART*, found there.

The existence of chromosomal homologies between *C. capitata*, *B. oleae* and *B. tryoni* as well as chromosome homologies between *C. capitata* and *D. melanogaster* further supports the concept that chromosome elements maintain their essential identity, not only within the genus *Drosophila* but also among distantly related Diptera despite the long divergence time. In the present study, similarities between *A. ludens* and *C. capitata* have been

Figure 36. Reference map of chromosome VI, sections 85-100.

identified although these were not especially extensive. However, they are sufficient to propose homology for four chromosomes of the two species. This has to be confirmed using other approaches such as *in situ* hybridization. Furthermore, *in situ* hybridization has been used to establish homologies between *D. melanogaster* and *C. capitata* and also confirmed previously proposed homologies between *C. capitata*, *B. oleae* and *B. tryoni*.
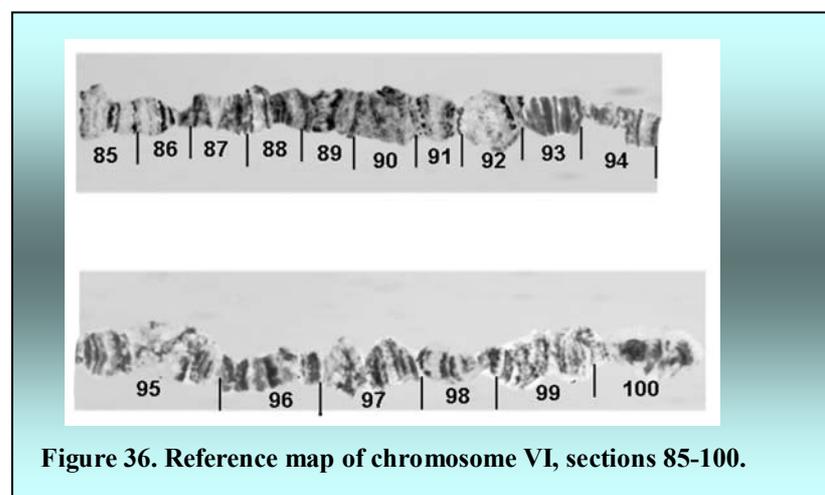
The results of the present study show that, in spite of the difficulties to work with *A. ludens* salivary gland polytene chromosomes, polytene maps with sufficient quality can be constructed and these are suitable for further cytogenetic analyses of this species. These maps could also be used as reference for the analysis of other *Anastrepha* species, most of which are also very serious fruit pests, but for which currently only very limited cytological data are available.

Colleagues at the Moscafrut facility in Tapachula, Mexico are now using these maps to analyze potential genetic sexing strains based on male-linked translocations and pupal colour mutations.

3.3.2. *Small Scale Evaluation of an Oxitec Genetic Sexing Strain OX3376B*

Oxitec is a Biotech company in the UK which has been at the forefront of developing improved strains of insects for use in SIT programmes. The Unit has collaborated with the company in order to assess the relative merits of one transgenic genetic sexing strain in comparison with the two genetic sexing strains currently in use in Mediterranean fruit fly SIT programmes. This included (1) an analysis of the behaviour of the strain under mass-rearing conditions, (2) mating competitiveness evaluation in field cages, and (3) a detailed production analysis of the Oxitec strain in comparison with non-transgenic genetic sexing strains under carefully controlled laboratory rearing.

3.3.2.1. *Colony production*

The transgenic strain, OX3376B, was developed by Oxitec and it carries the tetracycline-repressible auto regulatory tetO/tTA system. No additional effector gene is required because of the lethal side-effects of the tTA protein at sufficiently high concentrations and lethality is made female-specific by including the appropriate splice sites from the *C. capitata transformer* gene. In the presence of tetracycline (or its analogues) the system is switched off, no tTA is produced and the strain can be maintained with no lethality in males and females. The tetracycline analogue, chlortetracycline (CTC) was provided in the adult food or drinking water and in the larval diet; in both cases at a concentration of 100 μg/mL. In order to induce female lethality for male only production, CTC was omitted from the larval diet. However, to maintain the strain for colony production CTC is required in the adult diet in order to prevent females dying (**Figure 37**). However, the female-specific lethality shown in Figure 7 begins late and would not affect mass rearing efficiency as the production cages are maintained only for 10 to 14 days.

OX3376B was reared for 12 generations in parallel to the wild type strain EgII under standard rearing conditions. Each generation was set up with 34 ml of pupae in a 24 x 33 x 30cm cage. To measure the viability several samples of 100 eggs were collected per generation. In total over 12 000 eggs were used for each strain. The values determined were egg hatch, number of pupae, and number of males and females. These parameters are not only highly relevant for measuring the productivity of a strain but also can be used to determine the genetic behaviour
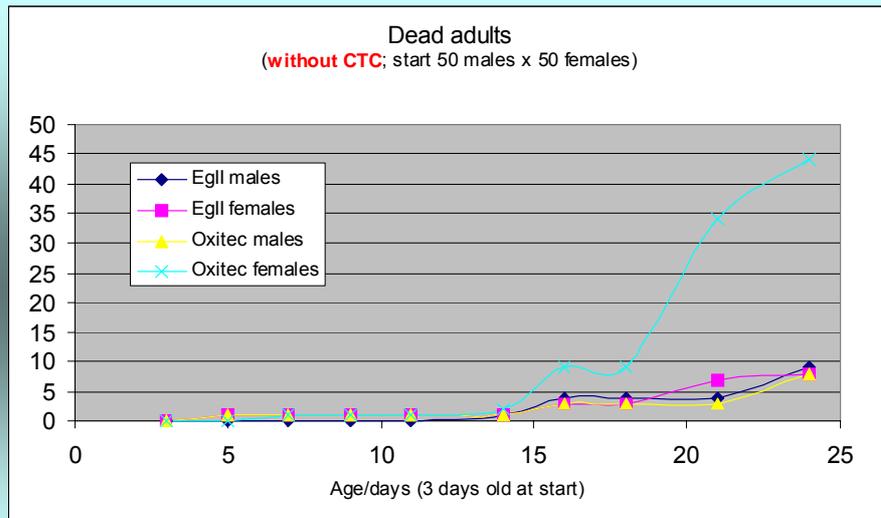
Figure 37. Adult longevity of OX3376B and EgII strains without CTC.

of a strain, e.g. at what developmental stage chromosomal rearrangements or transgenes cause lethality. Furthermore, these parameters are measured as part of the routine quality control process in all mass rearing facilities. The same rearing protocol has also been used for the evaluation of all sexing strains that were generated via classical genetics, e.g. VIENNA 7 and 8. In all cases a wild type strain, EgII, served as a control. The parameters measured here are extremely reproducible and show very low variability from experiment to experiment. A considerable amount of data for various types of strains (different wild type, different mutations, different chromosome rearrangements) have been accumulated over the years providing us with a solid base for the comparison of any new strains. However, as an additional confirmation to ensure that the analysis of OX3376B is directly comparable with earlier data sets for VIENNA 7 and 8, the wild type strain was reared in parallel under the same conditions.

Adult recovery from the four strains, VIENNA 7 and 8, EgII, and OX3376B, under colony production conditions is shown in the left part of **Figure 38**. The results indicate that the rearing conditions are close to optimal for EgII as this strain produces, in the absence of CTC, nearly the biological maximum, i.e. from 1000 eggs 868 fully emerged and non-deformed adults are produced. If CTC is included in the diet the
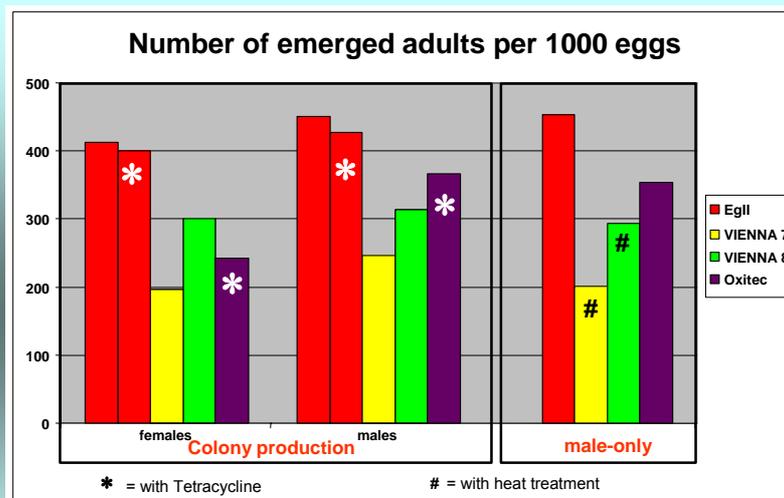
Figure 38. Productivity of colony rearing (left) and male only production (right). Value for EgII in male only production is theoretical.

	Percent hatch	Percent pupae from hatched eggs	Percent females from pupae	Percent males from pupae
EgII	92	98	46	51
VIENNA 7	82	65	37	46
VIENNA 8	81	86	43	45
Oxitec (CTC)	83	93	31	48

Figure 39. Comparison of production values obtained during colony rearing.

recovery is reduced only slightly to 829 adults per 1000 eggs. Overall the OX3376B strain produced when reared on CTC ca. 28% less adults than the EgII strain without CTC (868 versus 626 adults). This is largely due to a reduced recovery of females in the OX3376B strain. The OX3376B strain produced 253 females (38% less as compared to EgII) and 373 males (19% less as compared to EgII). In comparison to VIENNA 8, OX3376B produced 10% fewer females.

The female lethality in the OX3376B strain, in comparison with EgII (**Figure 39**) occurred during both the embryonic stage (the egg hatch is reduced significantly (83% versus 92%)) and during the pupal stage (i.e. female emergence was reduced significantly from 46% to 31%). Pupal recovery (93% versus 98%) and male emergence (48% versus 50%) were reduced only slightly.

The productivity of the OX3376B strain was also measured over time, i.e. depending on the age of the parents (**Figure 40**) and compared with EgII. The productivity of the EgII strain remained unchanged over time.
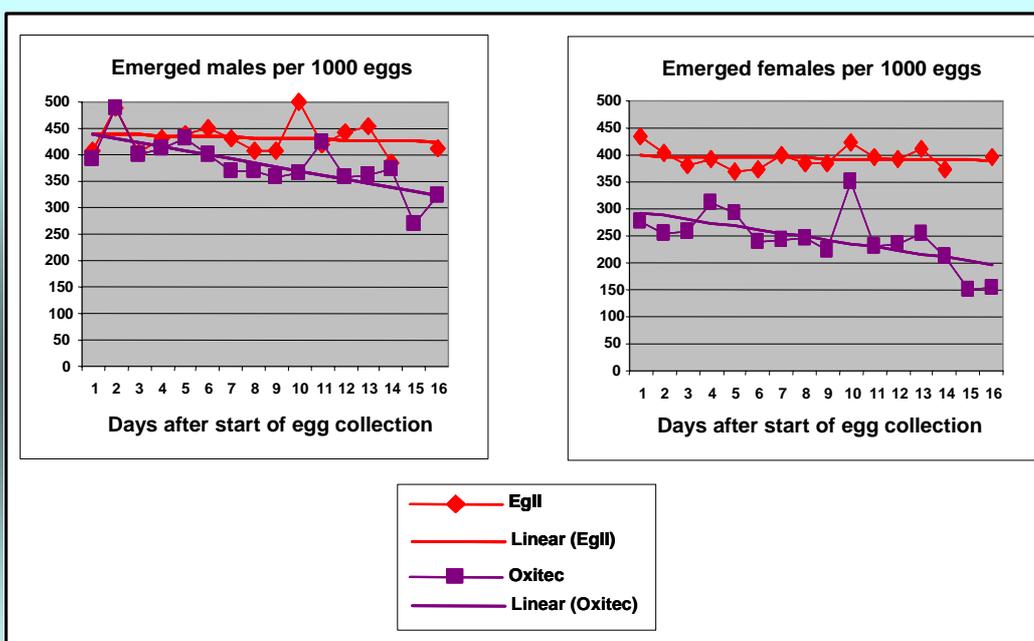
Figure 40. Male and female production correlated with age of parents. Only fully emerged and non-deformed adults considered.

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However, the productivity of OX3376B decreased slightly as the flies were getting older with the most significant reduction being in egg hatch. Other parameters such as pupation and female emergence also declined to some extent. There is no equivalent data for VIENNA 7 and 8.

It is more complicated to compare the two genetic sexing strains (GSS) with OX3376B. A GSS carries a Y-autosome translocation and the segregation behaviour (alternate versus adjacent-1 segregation) of this rearrangement determines the percentage of genetically unbalanced, and therefore usually inviable offspring. The developmental stage where this genetic lethality occurs differs between the two types of adjac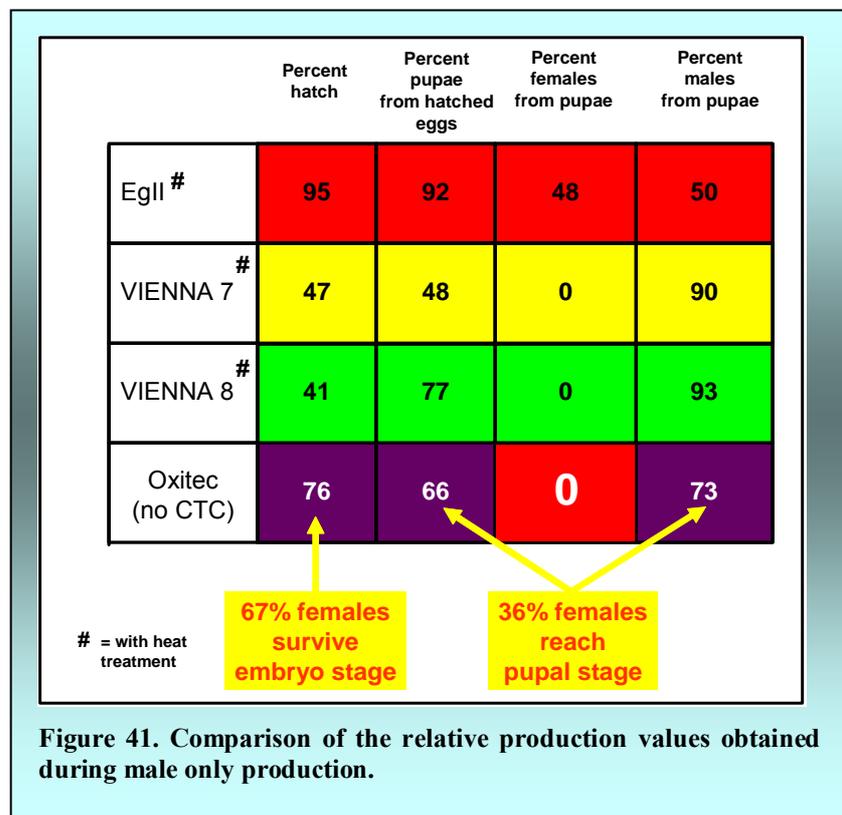ent-1 offspring. Offspring carrying a deletion die as embryos while offspring carrying a triplication die during later stages depending on the length of the triplication and the sex. VIENNA 7 and 8 are similar in the sense that both produce female triplication type adjacent-1 offspring that die during larval stages. The difference between the two GSS is that VIENNA 8 shows considerably more alternate segregation during male meiosis than VIENNA 7 with the consequence that the production of genetically balanced offspring is higher. This is clearly visible in **Figure 41**. VIENNA 8 shows an equal proportion of male and female adults and produces slightly more females (21%) and slightly less males (13%) than the OX3376B strain. Compared to EgII the reduced egg hatch and the lower pupal recovery are the consequence of the inviability of the adjacent-1 offspring (**Figure 41**).

3.3.2.2. Male-only production

Only males are produced when incubating the eggs of the strains VIENNA 7 and 8 for 24h at 34°C. To produce males in the OX3376B strain the larvae are reared on a diet without CTC. Compared to the theoretical number of males that would be produced by EgII all three sexing strains produce significant less (**Figure 38**, right): VIENNA 7, 56% less, VIENNA 8, 36% less and OX3376B, 23% less. OX3376B produces 18% more males per 1000 eggs than VIENNA 8. **Figure 41** shows the detailed results for the different life stages. VIENNA 7 and 8 show the expected lethality of the adjacent-1 offspring but produce pupae that are more or less free of unbalanced karyotypes as indicated by the very high adult emergence, i.e. a value very close to the wild type strain. In OX3376B the lethality of the females is relatively late. It can be calculated that a large

	Percent hatch	Percent pupae from hatched eggs	Percent females from pupae	Percent males from pupae
EgII #	95	92	48	50
VIENNA 7 #	47	48	0	90
VIENNA 8 #	41	77	0	93
Oxitec (no CTC)	76	66	0	73

= with heat treatment

67% females survive embryo stage

36% females reach pupal stage

Figure 41. Comparison of the relative production values obtained during male only production.

proportion of the females die as late as the pupal stage resulting in a very low adult emergence of 73%. However, in none of the experiments were any females observed indicating that the sexing system is very accurate and stable over the testing period of ca. 12 months.

A principle difference between the VIENNA strains all other sexing strains not based on the *temperature sensitive lethal* mutation (*tsl*), and the *white pupae* mutation (*wp*), irrespective if they are transgenic or conventional. The *tsl* mutation causes a slight delay in development of the homozygous females that is particularly apparent during pupal collection. The wild type $wp^+ tsl^+$ males pupate earlier than the *wp tsl* females. This characteristic allows the sex ratio in the rearing cages to be manipulated even without separating white and brown pupae via a sorting machine. For example, to increase egg production the cages of the male-only production step of the Filter Rearing System can be stocked with up to five times more females than males. This is not possible with other strains. **Figure 42** shows two comparisons of the strains evaluated here in terms of females produced/100 flies. In the first case the cages are stocked with equal numbers of pupae without changing the sex ratio. In this scenario cages with the OX3376B strain would contain 16% fewer females than cages with the EgII strain and produce in total 16% less flies and 8% less females. The VIENNA 7 strain has 7% less females than EgII and VIENNA 8 strain has 3% more females, however when the sex ratio is changed to 5:1 in favour of females both strains have about 75% more females than EgII and OX3376B contain 52% fewer females than in the VIENNA strains. This ability to manipulate the sex ratio in the VIENNA strains doubles their productivity in comparison with this particular transgenic sexing strain but this advantage would probably apply to all other strains as indicated above (**Figure 42**).

Figure 42. Number of females produced/cage under two scenarios: 1) cages filled with equal numbers of flies without manipulating the sex ratio (coloured columns) and 2) cages of Vienna strains filled with 5:1 ratio in favour of females (white columns. The percentages for the VIENNA strains (red) are given relative to EgII. The percentage given for OX3376B (yellow) is given relative to the VIENNA strains assuming the higher female stocking density. If these numbers are combined with the number of males/1000 eggs (Figure 38), it can be calculated that OX3376B produces 45% fewer males than VIENNA 8. It has to be stressed that this advantage of the VIENNA strains is specific for strains that utilize the *wp-tsl* sexing system, i.e. any strain, whether generated by Medelian genetics or via molecular strategies, that is not based on these selectable markers will not show this benefit.

3.4. Mosquito Rearing

3.4.1. Mass Production

3.4.1.1. Production planning

At the Unit, nearly every process in the rearing of *Anopheles arabiensis* is being given some attention though the resources are insufficient to develop each in as much depth as is needed. Fortunately, for many steps, methods (but not suitable equipment) have been devised previously or should be simply accomplished, e.g. a dosifier for larval diet slurry. However, even the simple steps require testing after initial prototyping and description. **Figure 43** provides an overview of the different components in the whole rearing and sterilization process.


Figure 43. Flow chart and activities during mosquito mass rearing.

Considerable effort was given to developing numerical estimates of the number of trays, cages, floor space, etc. required for the mass-production of one million males per day. As the process is currently envisioned, the Filter Rearing System will be used to maintain the integrity of the genetic sexing strain described in last year's report (GSS-V1, formerly named 5-33). This will require a one-generation amplification of the colony before the release generation is reared to produce only males. **Table 1** provides an overview of the overall requirements for the filter, amplification and release colonies.

Table 1. Sample calculations for facility and equipment planning purposes. Equipment corresponding to that needed for production is in the process of design and construction.

Elements	Filter	Amplification	Release
Duration of pupation (days)	3	3	2
Larvae at pupation (no.)	14632	296296	2100000
Larvae per tray (no.)	3000	3000	3000
Trays pupating (no.)	5	99	700
Area tray reqd. (2cm^2 per L4)	6000	6000	6000
Vol. per tray at 1 ml per L4 (ml)	3000	3000	3000
L2 to pupa duration (days)	7	7	7
L2-pupa trays in operation (no.)	22	428	3850
Trays per stack (no.)	50	50	50
Tray depth (cm)	1.7	1.7	1.7
Stack height (m)	0.85	0.85	0.85
Larvae per stack (no.)	150000	150000	150000
Space factor per rack	2.5	2.5	2.5
Area per rack (m^2)	1.5	1.5	1.5
Stacks in operation (no.)	1	9	77
Area for larval production (m^2)	1.5	13.5	115.5
Eggs needed per day (no.)	18064	365798	7407407

3.4.1.2.   *Larval density experiments*

Mosquito larvae develop in habitats whose resources – primarily diet – determine their growth rate, survival and size. The larval stage is also the stage during which density dependent effects are most likely to be evident. In the context of an SIT project, both density and diet amount are essential parameters to optimize in mass-production. In order to address these parameters, the development characteristics of *An. arabiensis* larvae under various laboratory conditions were analyzed. Because results of competition will be evident only under certain conditions, the boundary conditions of larval and diet density in which development is

Figure 44. Effect of larval density and diet on three life table parameters for *A. arabiensis*.
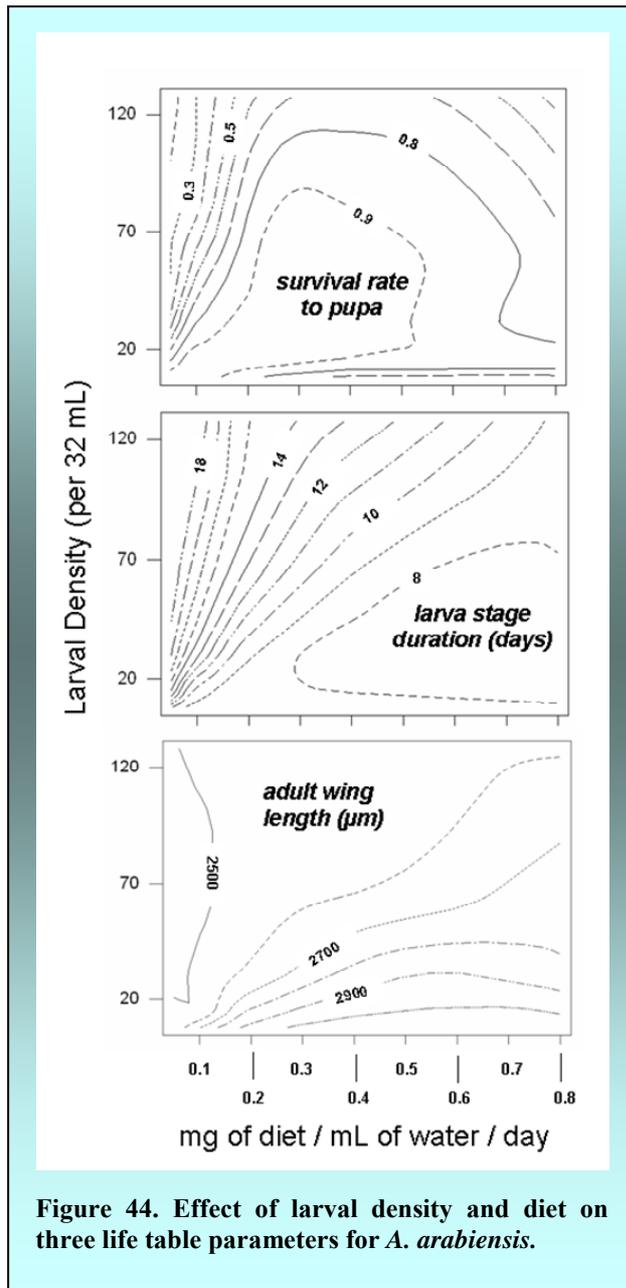
possible were determined. Survival rates, larval stage duration and adult size were determined as indicators of competition effects. These results will serve as an approximation for refining the production of larvae using the large tray system described below. Using a model rearing system and a complete factorial design, the conditions under which survival, development rate and size are maximal have been identified. Wing length and development rate are strongly correlated and appear to be resource dependent over much of the range tested (**Figure 44**).

These experiments were conducted using an artificial rearing system (polystyrene Petri dishes) which reveals potential biological responses of individuals and populations to variation in diet and larval density (**Figure 45**). Petri dishes contained trial diet components including beef liver powder, squid liver powder (powdered squid entrails), tuna meal, brewer's yeast, commercial aquaculture diets, and spirulina. Components and additives such as fatty acids and vitamins will be tested in various combinations after preliminary trials are completed.

The design of such experiments is complex due to the interacting effects of diet amount, concentration and larval density per mL of water and per cm^2 of surface area. For example, is the development of one larva with X weight of diet the same in twice the volume of water? If the concentration of diet is held constant, does changing the total volume affect development and over what range? Similar experiments have been conducted for various purposes many times but these experiments tested a wide range of conditions but minimized the number of variables in order to clearly understand the interactions.

3.4.1.3. Diet development

Mosquitoes have been cultured using numerous larval diets whose usefulness has been determined by consistent production of mosquitoes for colony maintenance purposes. Cost and male performance characteristics relevant to mating performance have rarely been considered. Males being used for release must be capable of mating competitively, an activity which involves dispersal capacity and possibly having a particular size. In this context and

given that much of the potential adult performance is determined by the diet provided in the larval stage, it is important to select diets which not only allow colony maintenance, but that provide necessary components for adult performance including longevity and flight performance.

Attention has been focused on diets that are inexpensive, available globally and can be obtained in consistently high quality. Special attention is given to controlled additives including antioxidants, eicosapentanoic acid, highly unsaturated fatty acids and vitamins. With a promising subset of these diets, competitive mating studies will be performed to determine whether there is a correlation between diet composition and competitiveness. A promising simple assay for flight performance is now being evaluated that can be conducted routinely in a mass-rearing facility.
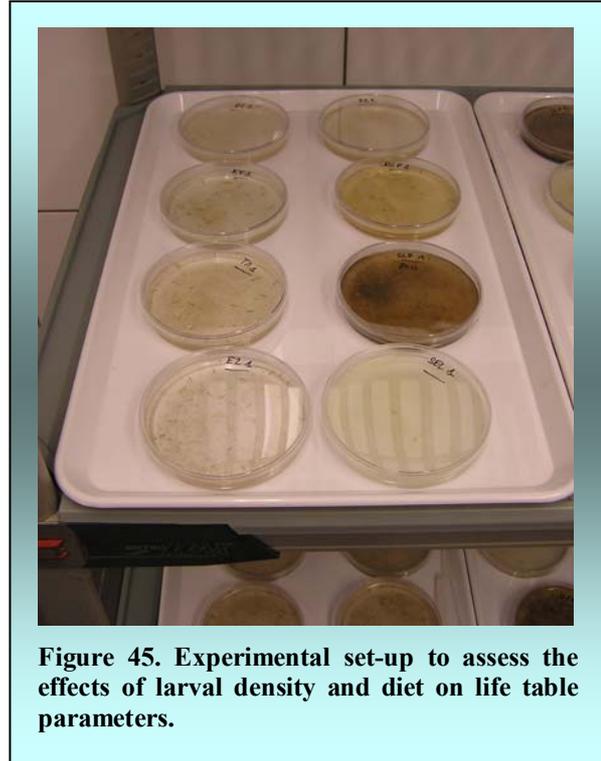

Figure 45. Experimental set-up to assess the effects of larval density and diet on life table parameters.

3.4.2. Mass Production Methods

3.4.2.1. Effects of cold storage on pupae and adults

Mass-production and release may be facilitated using methods to anesthetize pupae and/or adults for irradiation and transport and experiments have been conducted on cold storage of both stages. The initial experiments on pupal chilling at various temperatures for 24h demonstrated that temperatures sufficiently low to prevent eclosion also resulted in unacceptable mortality. However, the use of shorter periods of chilling that immobilize pupae sufficiently for irradiation and transport may be possible. Adult chilling was more promising and after 24h at 2-4°C approximately 20% mortality was observed relative to controls
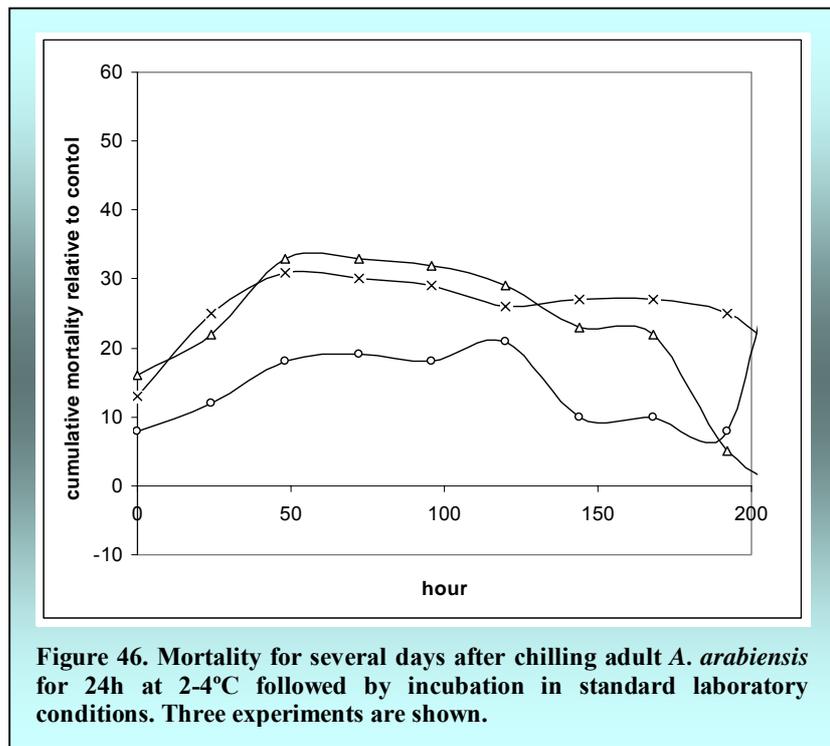

Figure 46. Mortality for several days after chilling adult *A. arabiensis* for 24h at 2-4ºC followed by incubation in standard laboratory conditions. Three experiments are shown.

(**Figure 46**). However, no special cold hardening treatment was given to the mosquitoes which might improve their survival and it is hoped that adult cold storage will be a useful alternative if it is required.

3.4.2.2. Mass-rearing tray design

Mosquito larvae need an aquatic environment to complete their development. Unlike other mosquito groups, larvae in the *anopheline* subfamily do not have a respiratory siphon but orient themselves in a parallel position on the water surface to breathe; they occasionally dive to the bottom to scavenge for food, but generally remain close to the water surface.

The rearing trays being designing will be an essential component of a rearing system that takes into account the larvae's requirement for space and feeding behaviour. Other important considerations when designing the rearing trays included the ease of handling, the availability of raw materials (when parts become damaged and need to be replaced), the cost of materials and the labour cost to build the trays.

A prototype tray made of thermoformed 5 mm thick ABS plastic has been produced (**Figure 47**). The tray was designed by the group and was further refined and produced by Glimberger Kunststoffe in Vienna. The close spacing of the trays will allow compact mosquito culture in order to minimize the amount of floor space required and to make handling more efficient. The tray has an external dimension of 60 x 100 cm and a thickness of 3.5mm. Based on available literature, it appears that a surface area of 2 cm^2 and a water depth between 0.5-2.5 cm is adequate for each larva. Based on this parameter it should be possible to culture 3000 larvae per tray. The larval density in this case will be about 1 larva per 1mL using 3L of water. The tray is designed to be drained by tilting it 15 degrees and will not be relocated within the factory while it contains water. Trays will be stacked and tilted to remove and collect larvae and pupae.

To create water movement in the central part of the tray during the draining process, symmetric slopes run toward the middle. Two other slopes of 15 degree angle were created at both ends of the tray to direct the flow of water towards the dedicated lips. This inclination permits the complete discharge of water by tilting one end of the tray approximately 25 cm. A ridge, 72 cm long and 3 cm high, is located in the centre of the tray. The purpose of this ridge is to give the tray additional structural stability and to increase the resting space available to the larvae. In addition, by drilling one or more holes in the ridge, an overflow system is created. A stack of trays will be filled using a water inlet at the top in a cascade. To avoid a direct flow of water from tray to tray before filling, a plastic plate will guide water overflowing from one tray away from the outlet of the tray below.
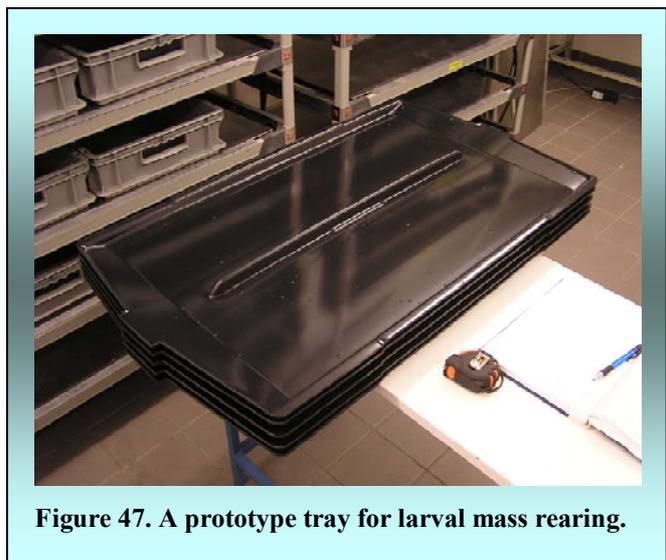

Figure 47. A prototype tray for larval mass rearing.

3.4.2.3. Holding rack

The rearing trays will be stacked in a rolling steel rack that holds 40-50 trays. The shape of the tray permits a 3cm distance between the levels. Using a tray of 60 x 100 cm, every rack is expected to produce 120 000-150 000 mosquitoes. The total height of the rack will be 160-190 cm. The rack can be lifted on one side to collect the larvae and pupae from the opposite side, where a plastic curtain will break their fall and direct them towards a catch tray at the bottom of the structure. Complete removal of larvae and pupae is not expected, so an operator will spray the

Figure 48.Concept design for tray tilting rack. A screw jack permits slow controlled elevation of one end of the trays so that drainage is controlled.

rack with water to remove the remaining individuals. The larvae and pupae will be collected in a basket, where they will be concentrated and filtered from the culture water in preparation for separation. Two designs to tilt the trays are currently being tested. The first design consists of a normal rigid stainless steel rack the entirety of which will be tilted mechanically (**Figure 48).** The second model includes an endless screw jack fixed on the rack which allows all trays to be lifted simultaneously by moving an inner frame connected to all trays. Unlike the first design described, this maneuver will move only the trays and will not require a movement of the main frame of the rack.
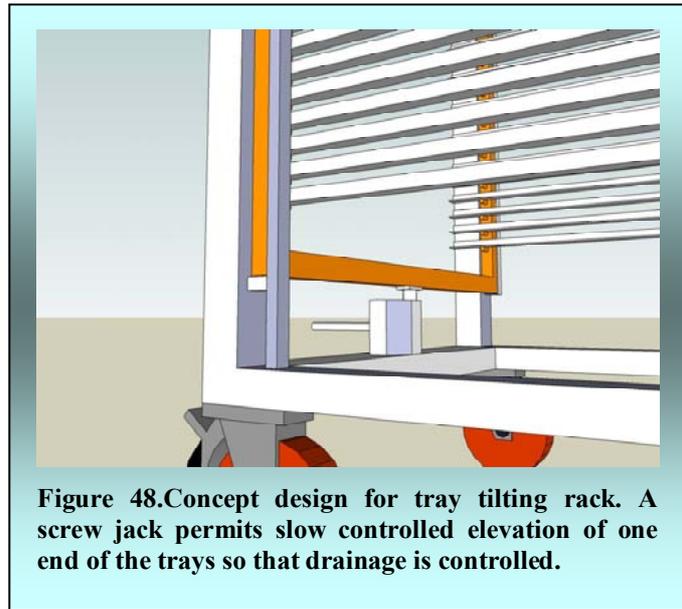
3.4.2.4.   Larva-pupa separation

After collecting the larvae and pupae from the rearing system, they need to be separated so that larvae can be re-introduced to the culture system to complete development and the pupae can be prepared for irradiation and release. The larvae must be uncontaminated by pupae so that excessive numbers of adults do not emerge into the facility. The goal is an unattended separation system that is capable of separating one million pupae in two hours.

Several methods to separate larvae from pupae have been tried in *An. arabiensis* and the method under development is based on the difference in buoyancy between the two. By creating a gentle vortex in a cylinder, the more buoyant pupae can be collected from the top and the more dense larvae are directed towards the bottom of the cylinder. The vortex is generated by a flow of water coming from a reservoir and is regulated by a flow valve and introduced tangentially into
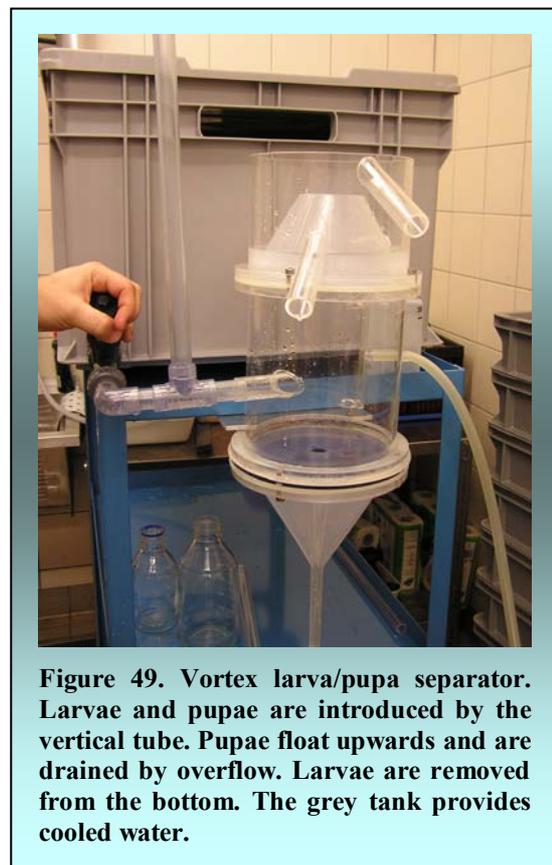

Figure 49. Vortex larva/pupa separator. Larvae and pupae are introduced by the vertical tube. Pupae float upwards and are drained by overflow. Larvae are removed from the bottom. The grey tank provides cooled water.

the chamber (**Figure 49**). To avoid a strong suction in the middle of the water column without reducing the movement of the water mass, a vortex breaker is placed at the bottom of the chamber. The addition of the breaker has notably improved separation, however, a small number of pupae can still be found with the larvae which will become problematic in a mass-rearing set-up. A very effective separation effect on larvae and pupae has been tested in the laboratory using ice-cold water, a method previously used extensively. However, it has been observed that cold shock produces stress and increases mortality after several exposures. Therefore, improvements were sought by reducing the stress through testing higher temperatures. After several trials, it was found that the highest temperature that still effectively separates larvae from pupae is 10ºC. No mortality was recorded at this temperature and separation was instantaneous. Currently, trials are being carried out to test whether the separation effects of the two methods can be combined to achieve an efficient separation device that is continuous, economical, easily constructed and maintained.
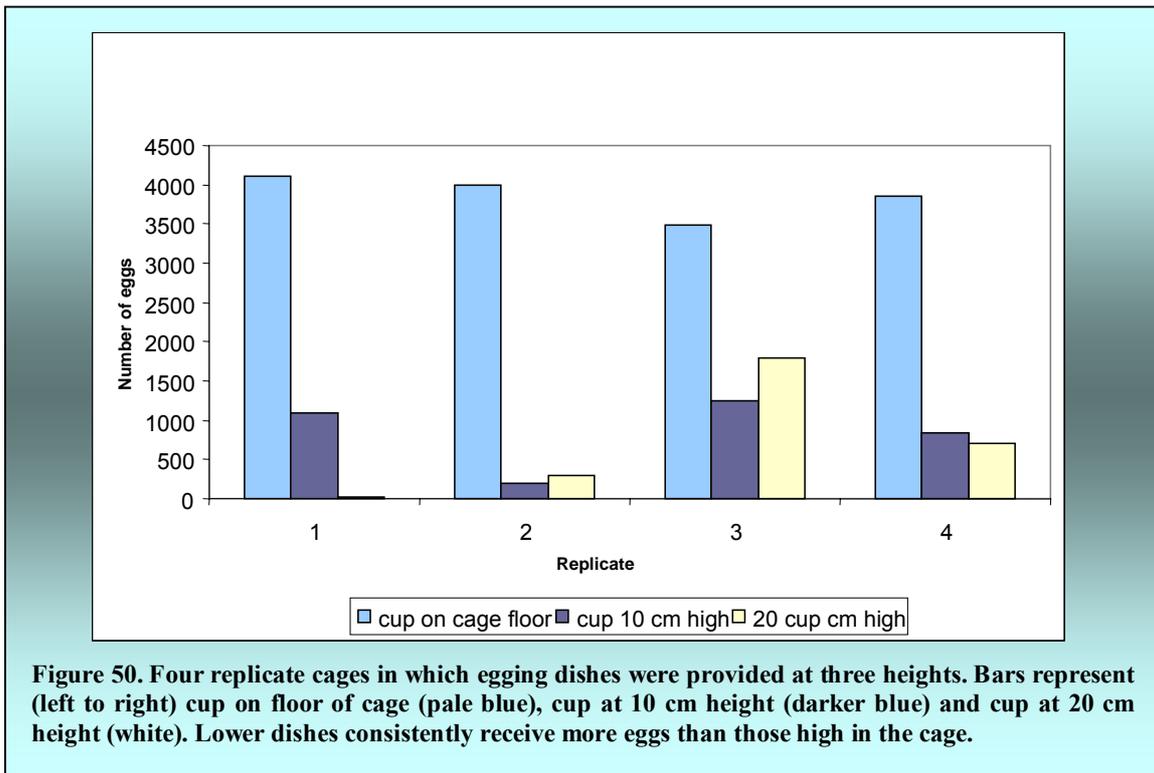
3.4.2.5. *Mass-production adult cage trials*

The mass-production cage (**Activities Report 2006**) is currently undergoing testing in Ghana, Seibersdorf, French Polynesia and Italy. In spite of extensive development, the Seibersdorf experience indicates that significant flaws in the design still exist. Of the four "appliances:" sugar feeder, blood feeder, adult emergence and egg collection, only the sugar feeder is operating satisfactorily on a routine basis. Given the demands of mass-production and the general redesign of the cage from the first prototype followed by a more conventional box type cage, all three deficient appliances are being redesigned. This is a significant technical setback, which may necessitate returning to simpler laboratory scale systems until the problems are solved.

The current blood feeding system that works by heating a collagen sausage in a metal mesh elicits poor response in terms of the number of females attracted to the blood source and the even smaller number of females that engorge. Preliminary tests have shown that soaking the membrane in water for 1h slightly improved feeding. This may be due to the increased permeability of the membrane that makes it more attractive and easier to penetrate. A more uniform heat distribution is needed to heat the blood source and this may require finding an alternative to the current heating device that uses metal mesh.

It has been conclusively shown that ovipositing females have a strong preference for dishes that are located at the bottom of the cage (**Figure 50**). The current oviposition tray is 8 cm high and does not provide a vertical support from where females can lay eggs. Tests are ongoing to modify the location and the shape of the oviposition tray.

3.4.3. *Continuing Screen for a Genetic Sexing Strain*

The isolation of the genetic sex separation strain GSS-V1 (**Activities Report 2007**) is appearing to be even more fortunate than was originally apparent. In spite of a continuing screen of approximately 600 families, no additional candidate GSS have been isolated. The existing screen is based solely on dieldrin resistance (*Rdl*) linkage to the Y chromosome, but this approach could be made more efficient by the availability of a second marker on the chromosome on which *Rdl* is located. A phenotypic marker, *collarless,* would be ideal for this purpose, but alleles for this mutation have not been detected in the colonies in the Unit. Other laboratories are being contacted to see if they have the mutant strain.

Figure 50. Four replicate cages in which egging dishes were provided at three heights. Bars represent (left to right) cup on floor of cage (pale blue), cup at 10 cm height (darker blue) and cup at 20 cm height (white). Lower dishes consistently receive more eggs than those high in the cage.

3.5. Stable Isotopes

3.5.1. Determining Mating Status in Mediterranean Fruit Flies Using Isotope Marking

Results reported in the **Activities Report 2007** suggested that it may be possible to directly measure the isotopic signature of the spermathecae of fruit flies and to follow the fate of transfer of C4 sperm from mass-reared and released sterile males into C3 spermathecae of wild females. This could possibly provide an opportunity to develop a method to estimate sterile insect insemination rates in the field. Therefore a second series of experiments was initiated and conducted in 2008 on male Mediterranean fruit flies reared on a C4 diet which were mated with female Mediterranean fruit flies fed on a C3 diet and sperm transfer followed by dissecting out the spermathecae and measuring the isotopic signature by isotope ratio mass spectrometry. Initial results on spermathecae indicated that it was not necessary to use a spike to achieve minimum detection limits in the mass spectrometer as the amounts of both carbon and nitrogen were above this limit, i.e. 5.96 µg and 31.41 µg, respectively. This made direct measurement possible. If the contribution of male sperm to the carbon mass of the spermathecae is greater than 20% then sperm transfer should be easily detectable.

Approximately 1000 adults were reared on either a C3 sugar beet based-diet or a C4 sugar cane based diet and a cohort of C4 males were mated with C3 females and vice versa. One hundred mating pairs were collected from each group, and after separation the females were isolated from the males. Spermathecae were dissected out of the females 1, 3, and 6 days after mating and analysed for isotopic signature, five replicates per sampling time. Flies were individually dried (60°C for 24h) and stored in eppendorf tubes containing a few grains of dry silica gel. Spermathecae were dissected out under a microscope and placed on a glass fibre disc. Samples were sealed into 8 mm x 5 mm tin cups and analyzed using a Carlo Erba (Milan, Italy) carbon nitrogen (CN) analyzer, linked to an Thermo Delta Advantage automated isotope ratio mass spectrometer (IRMS) (Bremen, Germany) and the isotopic signature $\delta^{13}C$‰ was calculated referenced to Vienna Pee Dee Belemite (VPDB).

Results from the C3 fed and C4 fed populations were not as isotopically distinct as expected probably due to the other components of the diet being from a C3 source, such as the bran and the brewers yeast. There was only about a 4.5‰ difference between the two populations compared to that expected i.e. 9‰,
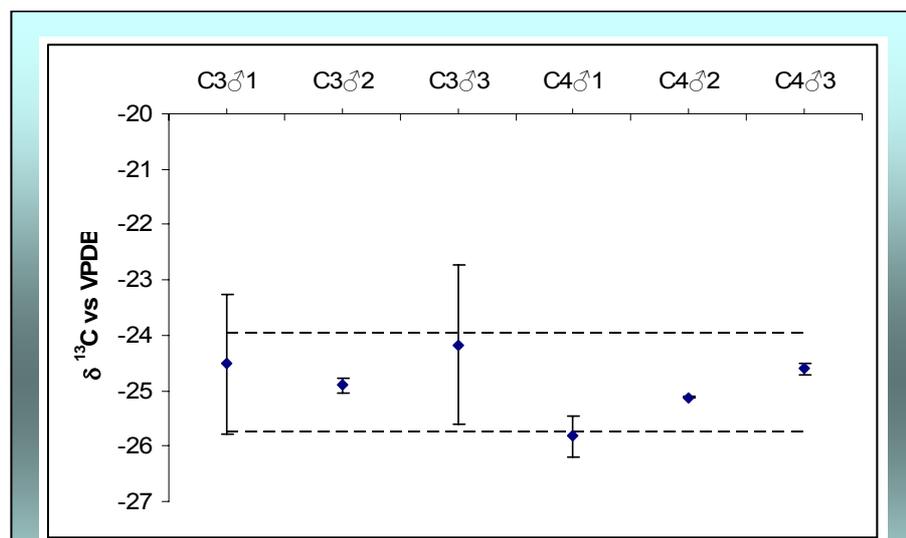

Figure 51. Mean isotopic signatures of C3 female spermathecae, mated with either C3 males or C4 males, at 1, 3 and 6 days after mating. Error bars plus and minus variance of the mean. Dotted lines are the modelled propagated error of the mixing model, using the mean C3 female and C4 male bulk isotopic values.
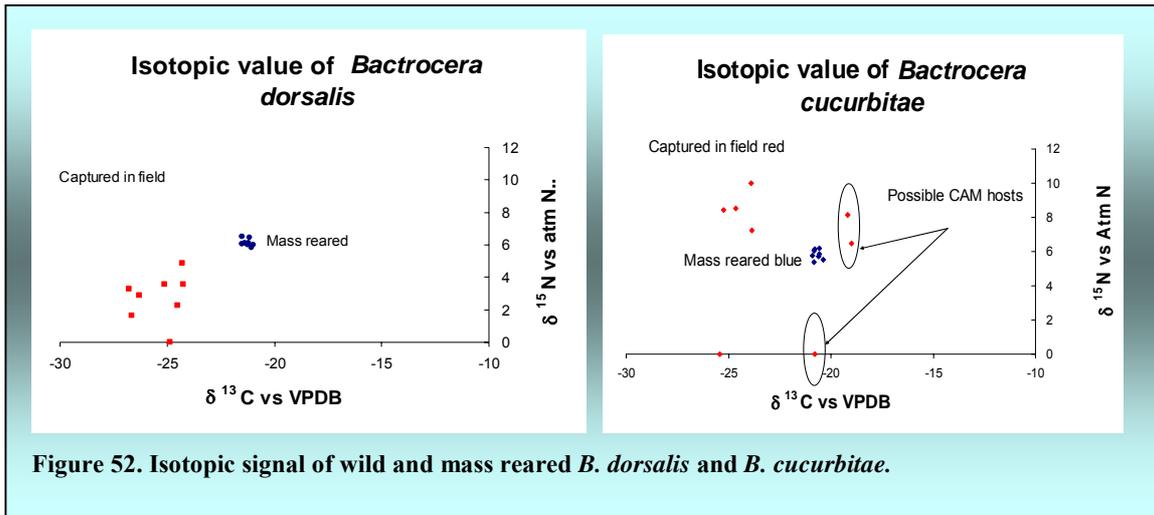
Figure 52. Isotopic signal of wild and mass reared *B. dorsalis* and *B. cucurbitae*.

making it impossible to detect sperm transfer from the C4 reared males to the C3 reared females (**Figure 51**). Modelling indicated that only when the male contributes over 60% of the carbon to the spermathecae is there a sufficient signal to detect above the propagated error. These results again confirm that a more distinct isotopic differentiation between the males and females is required.
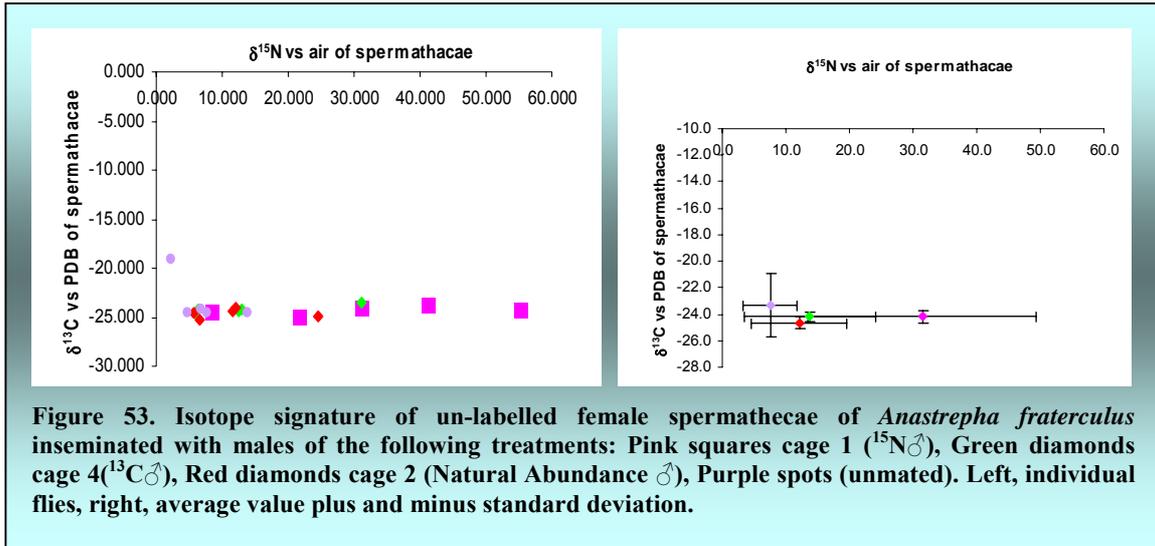
Although the results were disappointing it was shown that the spermathecae could be analyzed without a spike, which provides more resolution than with mosquitoes. Further experiments could include replacing bran and brewers yeast with material from a C4 source. Another possibility would be to microlabel the food source with enriched ^{13}C glucose or ^{15}N.

3.5.2. *Intrinsic Marking of* **Bactrocera cucurbitae** *and* **Bactrocera dorsalis**

Initial studies on distinguishing wild versus mass-reared *B. cucurbitae* and *B. dorsalis* populations using intrinsic isotope markers were also undertaken. The results (**Figure 52**) showed that the method worked well for *B. dorsalis* but there were problems with the *B. cucurbitae*. Following discussions with colleagues from Hawaii it was suggested that this may have been due to possible alternative host species for the *B. cucurbitae* such as the dragon fruit, genus *Hylocereus* (sweet pitayas), which are CAM (crassulacean acid metabolism) plants with isotopic values similar to C4 species.

3.5.3. *Detecting Sperm Transfer in* **Anastrepha fraterculus**

Larvae were reared in diet labelled either with ^{15}N or ^{13}C or on unlabeled diet. Seven day old males of each type were released into different field cages together with unlabeled females. Mating pairs were isolated as soon as detected and the mating start time was registered. The pair was placed in a container and checked every 5min until the pair disengaged, and the time noted to calculate the mating duration. Each pair was then frozen, and males and females were placed in a separate eppendorf tube with an identification number. The spermathecae were removed from females and testes from males and then analyzed for isotope signature. Overall it was clear that there was insufficient labelling of the males to reliably detect sperm transfer (**Figure 53**). Initial results suggested that the N marker was a more reliable marker; however there were some problems with carry over in the mass spectrometer on this day so these results should be approached with some caution.

Figure 53. Isotope signature of un-labelled female spermathecae of *Anastrepha fraterculus* inseminated with males of the following treatments: Pink squares cage 1 (¹⁵N♂), Green diamonds cage 4(¹³C♂), Red diamonds cage 2 (Natural Abundance ♂), Purple spots (unmated). Left, individual flies, right, average value plus and minus standard deviation.

3.5.4. *Isotopic Signal of Wild and Mass-Reared* Glossina pallidipes

Field samples, including both wild flies and released sterile males, and laboratory samples of *G. pallidipes* from Ethiopia were analyzed to determine whether there are sufficient isotopic differences between factory flies and wild fly populations which could be used as isotope intrinsic markers to compliment the fluorescent markers. Flies were dissected into legs, body and wings, and analyzed for ^{13}C and ^{15}N natural abundance levels. In addition samples of wings and single legs were sent to UC Davis, California for ^{18}O and deuterium analysis.

For the ^{13}C and ^{15}N analysis there appear to be no real differences in the isotopic signature between the three groups of flies in the body, wings or legs. The deuterium samples were highly variable with large standard deviations (**Figure 54**). This could have been due to the low sample size in comparison to the standards used, or it could reflect true differences in isotopic signature due to differences in flight activity or source waters. There was no consistent or distinctive isotopic difference
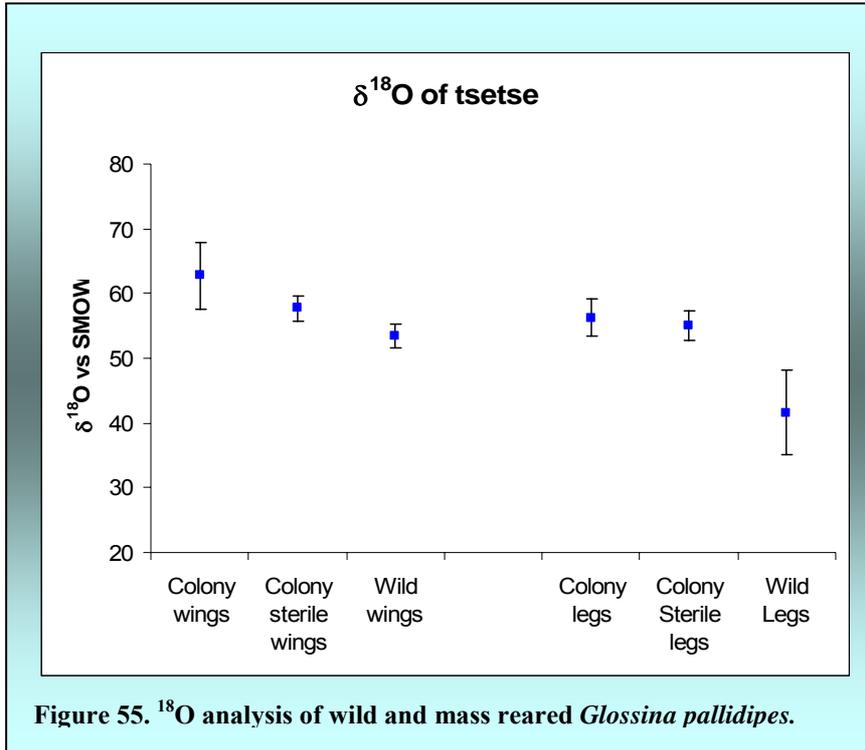

Figure 54. Deuterium analysis of wild and mass reared *Glossina pallidipes*.

55
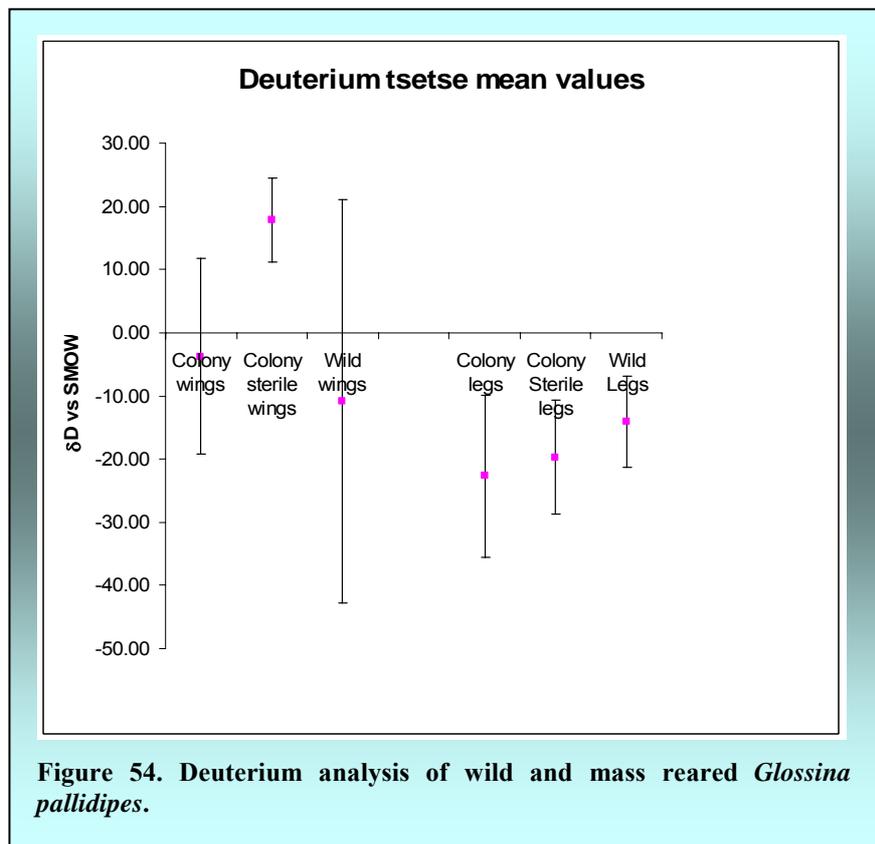
Figure 55. ^{18}O analysis of wild and mass reared _Glossina pallidipes._

between the factory and wild populations and there were no correlations between leg and wing values.

In contrast, the oxygen δ^{18}O values were statistically tighter showing less variation. There were significant differences between factory leg δ^{18}O values and wild leg values, a contrast which was also evident in the wing values but the distinction was not so great (**Figure 55**). The differences could be attributable to different signatures of the source water used. As the wild values are less enriched than the factory it seems to suggest that the factory water is derived from a reservoir which is subject to high evaporation rates. Although these could be useful in distinguishing populations further tests to establish the persistence of the difference would be required to ensure that a reliable conclusion on the origin of the fly is made. This is due to the fact that there will be turnover of tissues when the factory fly moves from a factory diet to wild diet and therefore factory signal may rapidly approach the wild signature.

3.5.5. Isotopic Signal of Wild and Mass-Reared Pectinophora gossypiella

In collaboration with Dr Gregory Simmons, USDA-APHIS-PPQ-CPHST, Phoenix, USA, the C values of field and mass-reared samples of pink bollworm _Pectinophora gossypiella_ were analyzed (**Figure 56**). There was no reliable distinction in the carbon isotope values, probably because only a small amount of sugar (C4) is used in the moth diet, however there appeared to be a some distinction in δ^{15}N values, which could have been a result of the high soybean content of the diet.
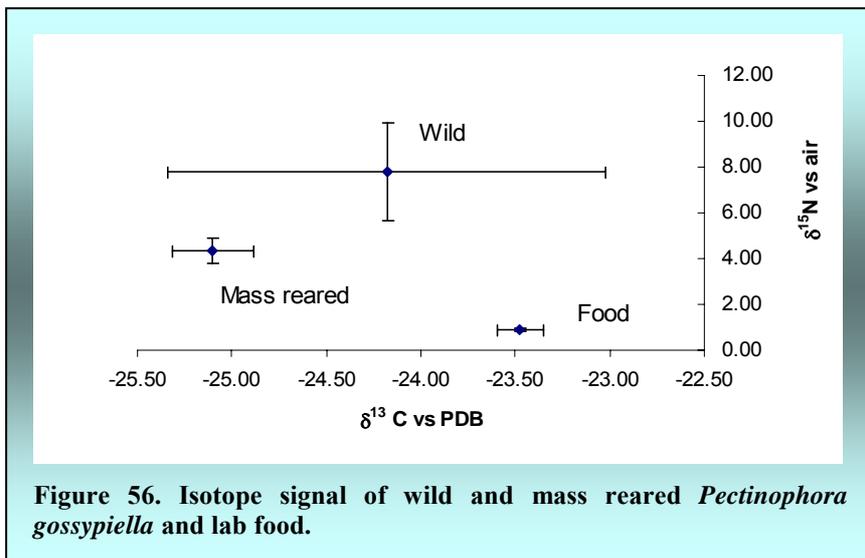
Figure 56. Isotope signal of wild and mass reared _Pectinophora gossypiella_ and lab food.

4. APPENDICES

4.1. Personnel Changes

In March 2008 Mr Marc Vreysen took over as Unit Head and Mr Andrew Jessup began his work as the leader of the fruit fly rearing and quality control group. The fruit fly group was joined in August by Ms. Dina Orozco, a cost-free expert from Mexico, who specializes in fruit fly mass rearing and quality control. Mr. Diego Segura left the group and returned to Buenos Aires, Argentina. In the tsetse group Mr. Patrick Abila was recruited to support the work on the tsetse virus and Mr. Daniel Briceňo returned to Costa Rica after a one year sabbatical. In the genetic sexing group Ms Antigone Zacharopoulou returned to Patras, Greece after a two year sabbatical. In the mosquito group, Ms Rosemary Burton returned to Oxitec in Oxford and the group was joined by Mr Jeremie Gilles and Mr Fabrizio Balestrino. Mr Alan Robinson was recruited to complete several documents and publications. Ms Michelle Helinski, who left the Unit in 2007 completed her PhD at the University of Wageningen and is now at Cornell University in New York.

4.2. Publications

ABD-ALLA, AMM, F COUSSERANS, **AG PARKER,** JA JEHLE, NJ PARKER, JM VLAK, **AS ROBINSON** and M BERGOIN (2008). Genome analysis of *Glossina pallidipes* salivary gland hypertrophy virus reveals a novel, large, double-stranded circular DNA virus. Journal of Virology **82**, 4595-4611.

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ABILA, PP, MA SLOTMAN, A PARMAKELIS, KB DION, **AS ROBINSON**, VB MUWANIKA, JCK ENYARU, LM OKEDI, S AKSOY and A CACCONE (2008). High levels of genetic differentiation between Ugandan *Glossina fuscipes fuscipes* populations separated by Lake Kyoga. PLoS Neglected Tropical Diseases **2**: e242.

BENEDICT, MQ and **AS ROBINSON** (2008). Impact of technological improvements on traditional control strategies. In: Transgenesis and Management of Vector-Borne Diseases, Ed. S. Aksoy, Landes Bioscience and Springer Science + Business Media LLC, New York, USA. pp. 84-92.

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BROWN, PJE, KJ KOMATSU, **PP ABILA, AS ROBINSON**, LM OKEDI, N DYER, MJ DONNELLY, MA SLOTMAN and A CACCONE (2008). Polymorphic microsatellite markers for the tsetse fly *Glossina fuscipes fuscipes* (Diptera: Glossinidae), a vector of human African trypanosomiasis. Molecular Ecology Resources **8**, 506-1508.

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DYER, NA, SP LAWTON, S RAVEL, KS CHOI. MJ LEHANE, **AS ROBINSON**, LA OKEDI, MJR. HALL, P SOLANO and MJ DONNELLY (2008). Molecular phylogenetics of tsetse flies (*Diptera: Glossina*) based on mitochondrial (*COI*, *16S*, *ND2*) and nuclear ribosomal DNA sequences. Molecular Phylogenetics and Evolution **49**, 227-239. doi: 10.1016/j.ympev.2008.07.011

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HELINSKI, ME; MM HASSAN, WM EL MOTASIM, **CA MALCOLM**, BG KNOLS and B EL SAYED (2008).Towards a sterile insect technique field release of *Anopheles arabiensis* mosquitoes in Sudan: Irradiation, transportation, and field cage experimentation. Malaria Journal **7**, 65.

HELINSKI, ME and BG KNOLS (2008). The influence of late-stage pupal irradiation and increased irradiated: un-irradiated male ratio on mating competitiveness of the malaria mosquito *Anopheles arabiensis* Patton. Bulletin of Entomological Research **9**, 1-6.

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4.3. Travels

4.3.1. Staff

Name	Destination	Period	Purpose of Travel
Abd Alla, Adly	Warwick, UK	3-7 August	Present a paper at the 41st annual meeting of the Society for Invertebrate Pathology and the 9th International Conference on *Bacillus thuringiensis*.
Balestrino, Fabrizio	Perugia, Italy	15-18 October	Assist in planning a mosquito production facility and semi-field cage.
Boigner, Rudolf	Majava, Slovakia	11 March	Observe blood collection and advise on improvements.
Benedict, Mark	La Reunion, France	12-15 February	Attend planning meeting for SIT research areas.
	London, UK	25-28 February	Participate in the planning of a mosquito SIT research proposal for the EU.
	Ghent, Belgium	17-20 March	Scientific Secretary of 2nd RM on "Development of standardised mass rearing systems for male *Anopheles arabiensis* mosquitoes.
	Panama, City, Panama Guatemala City, Guatemala	16-21 June	Evaluate mass rearing technology in relation to mosquito mass production.
	Seattle, USA	28-30 July	Attend Gates Foundation meeting on research and development for vector control.
	Khartoum, Sudan	3-7 August	Participate in a meeting to design a mosquito mass rearing facility.
	Perugia, Italy	15-18 October	Assist in planning a mosquito production facility and semi-field cage.

Name	Destination	Period	Purpose of Travel
	New Orleans, USA	7-11 December	Attend MR4 Scientific Advisory Committee meeting and attend American Society of Tropical Medicine and Hygiene meeting.
Franz, Gerald	Antigua, Guatemala	18-22 August	Scientific Secretary of the final RCM on "Molecular technologies to Improve the effectiveness of SIT".
			Scientific Secretary of a Consultants Meeting to review opportunities and requirements for implementing a CRP on "Assessing the Potential for improved strains of insect pest for SIT.
Jessup, Andrew	Valencia, Spain Palma de Mallorca, Spain	1-8 April	Attend RCM on Development of Mass Rearing for the New World (Anastrepha) and Asian (Bactrocera) fruit fly pests in support of SIT.
			To attend the 1st Tephritid Workers Meeting, Palma de Mallorca.
	Jerusalem, Israel	21-25 September	Review and discuss progress in TC ISR5012.
	Avignon, France	26-30 October	Present a paper at the 7th International Conference on Integrated Fruit Production.
Mohammed, Hasim	Majava, Slovakia	11 March	Observe blood collection and advise on improvements.
Parker, Andrew	Ouagadougou, Burkina Faso	21-24 January	Identify equipment needed for the establishment of a large scale mass rearing facility.
	Durban, South Africa	7-11 July	Present two papers at the International Conference of Entomology.
	Addis Ababa, Ethiopia	28 July-1 August	Review and discuss progress in TC ETH5012.

Name	Destination	Period	Purpose of Travel
	Addis Ababa, Ethiopa	13-17 October	Scientific Secretary of RCM on "Improved and harmonized quality control for expanded tsetse production sterilization and field application".
	Addis Ababa, Ethiopia	20-22 October	Review and discuss progress in TC ETH5012.
Vreysen, Marc	Addis Ababa, Arba Minch, Awassa, Ethiopia	22-28 January	Attend the Technical Advisory Group and the Steering Committee of the STEP and monitor progress in ETH5012.
	Tunis, Tunisia	11-13 February	Review status of date moth rearing and monitor progress in TUN5025.
	Dakar, Senegal	10-14 March	Participate in the Regional Training Course and monitor progress in the tsetse project SEN 5029.
	Durban, South Africa	6-11 July	Present two papers at the International Conference of Entomology.
	Dakar, Senegal	6-10 October	Monitor progress in SEN5029 and participate in a national workshop.
	Tororo, Kampala, Uganda	16-19 November	Attend the INCO meeting and monitor progress in UGA5027.
	Cotonou, Benin	24-28 November	To participate in the International Conference on Demographic and Climate Changes and its Impact on Vector-borne Diseases in West Africa and present a keynote paper.

4.3.2. Consultants

Name	Destination	Period	Purpose of Travel
Briceno, Daniel	Karlsruhe, Germany	25-30 August	Carry out high resolution imaging of tsetse mating at the ANKA Synchrotron
	Addis Ababa	13-17 October	Participate in RCM on "Improved and harmonized quality control for expanded tsetse production sterilization and field application".
Hood-Nowotny, Rebecca	Rabat, Morocco	1-3 July	Present a paper at the meeting Biotechnology for Locust Control.
Robinson, Alan	Antigua, Guatemala	18-22 August	Consultant for the meeting "Assessing the Potential for improved strains of insect pest for SIT.
	Mazatlan, Mexico	2-7 November	Present a paper at the 7th Meeting of the Working Group on Fruit Flies of the Western Hemisphere.

4.4. External Collaborations and Partnerships

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

PBARC, USDA-ARS, P.O. Box 4459, Hilo, HI, 96720, USA	Fruit fly liquid diet
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 Inst., University of Vienna, Althaustrasse 14, A-1090 Vienna, Austria	Tsetse sound production

4.5. Trainees, Fellows and Scientific Visitors

Name	Country	Duration	Topic of Training
Fellows			
Sayed, Mr WA	Egypt	12 months	Fruit fly rearing and cytogenetics
Changasi, Mr RE	Kenya	6 weeks	Tsetse rearing and virus analysis
De Beer, Ms C	South Africa	3 months	Tsetse rearing
Memon, Mr RM	Pakistan	4 months	X-ray biological dosimetry
Palma Cabrera, Mr JJ	Chile	1 month	X-ray biological dosimetry
Mastrangelo, Mr TD	Brazil	4 months	X-ray biological dosimetry
Mhindurwa, Mr A	Zimbabwe	5 days	Radiation dosimetry
Katjimune, Mr NE	Botswana	5 days	Radiation dosimetry
Scientific Visitors			
Walder, Mr JMM	Brazil	5 days	X-ray dosimetry
Reis, Ms V	Brazil	10 days	X-ray dosimetry

4.6. Co-ordinated Research Projects (CRP) and Technical Cooperation Projects (TCP)

CRP Title	Scientific Secretary
Application of Established Molecular Technologies to Improve the Effectiveness of SIT (2003-2008).	Franz, Gerald
Development of Mass Rearing in Support of *Anastrepha* and *Bactrocera* Fruit Fly SIT (2004-2008).	Jessup, Andrew
Product and Process Quality Control for Standardization of Tsetse Mass Production, Sterilization and SIT Release (2003-2008).	Parker, Andrew
Improving SIT for Tsetse Flies Through Research on their Symbionts and Pathogens (2007-2013).	Abd-Alla, Adly
Mass Rearing and Pre-Release Biology of *An. arabiensis* (2005-2009).	Benedict, Mark
Post Release Biology of *Anopheles arabiensis* (2008-2012)	Benedict, Mark
Increasing the Efficiency of SIT for Lepidopteran Pests by Enhanced Quality Control (2009-2013).	Vreysen, Marc
TCP Title	**Technical Officer**
Investigating the Use of the Sterile Insect Technique for Controlling Mosquitoes in Northern Sudan.	Benedict, Mark
Implementing the Pre-Operational Phase to Create a Zone Free of *Glossina palpalis gambiensis* Using the Sterile Insect Technique (SIT)	Vreysen, Marc
Supporting the Creation of a Tsetse-Free Zone in Southern Mozambique and North-East South Africa	Vreysen, Marc
Assessing the Use of Inherited Sterility as a Genetic Control Method against the Carob Moth	Vreysen, Marc
Strengthening the Capacity to Use the Sterile Insect Technique for the Olive Fruit Fly.	Jessup, Andrew
Developing Integrated Control of the Olive Fruit Fly.	Jessup, Andrew

4.7. Abbreviations

ABS　　　　Acrylonitrile Butadiene Styrene

Bd　　　　*Bactrocera dorsalis*

Bi　　　　*Bactrocera invadens*

BOKU　　　University for Natural Resources and Applied Life Systems

CAM　　　Crassulacean Acid Metabolism

CRP　　　Coordinated Research Project

CTC　　　Chlortetracycline

DNA　　　Deoxyribonucleic Acid

DUR　　　Dose Uniformity Ratio

EU FP7　　European Union Framework Programme 7

FEY　　　Fresh Egg Yolk

GSS　　　Genetic Sexing Strain

IEY　　　Industrial Egg Yolk

M　　　　Methoprene

mRNA　　messenger RNA

NIST　　　National Institute for Standards and Technology

ORF　　　Open Reading Frames

P　　　　Protein

PCR　　　polymerase chain reaction

PH　　　　Protein Hydrolysate

qPCR　　　Quantitative PCR

RNA　　　Ribonucleic acid

RNAi　　　RNA interference

S　　　　Sugar

SGHV　　Salivary gland hypertrophy virus

GpSGHV　*Glossina pallidipes* salivary gland hypertrophy virus

SIT　　　Sterile insect technique

W　　　　Water