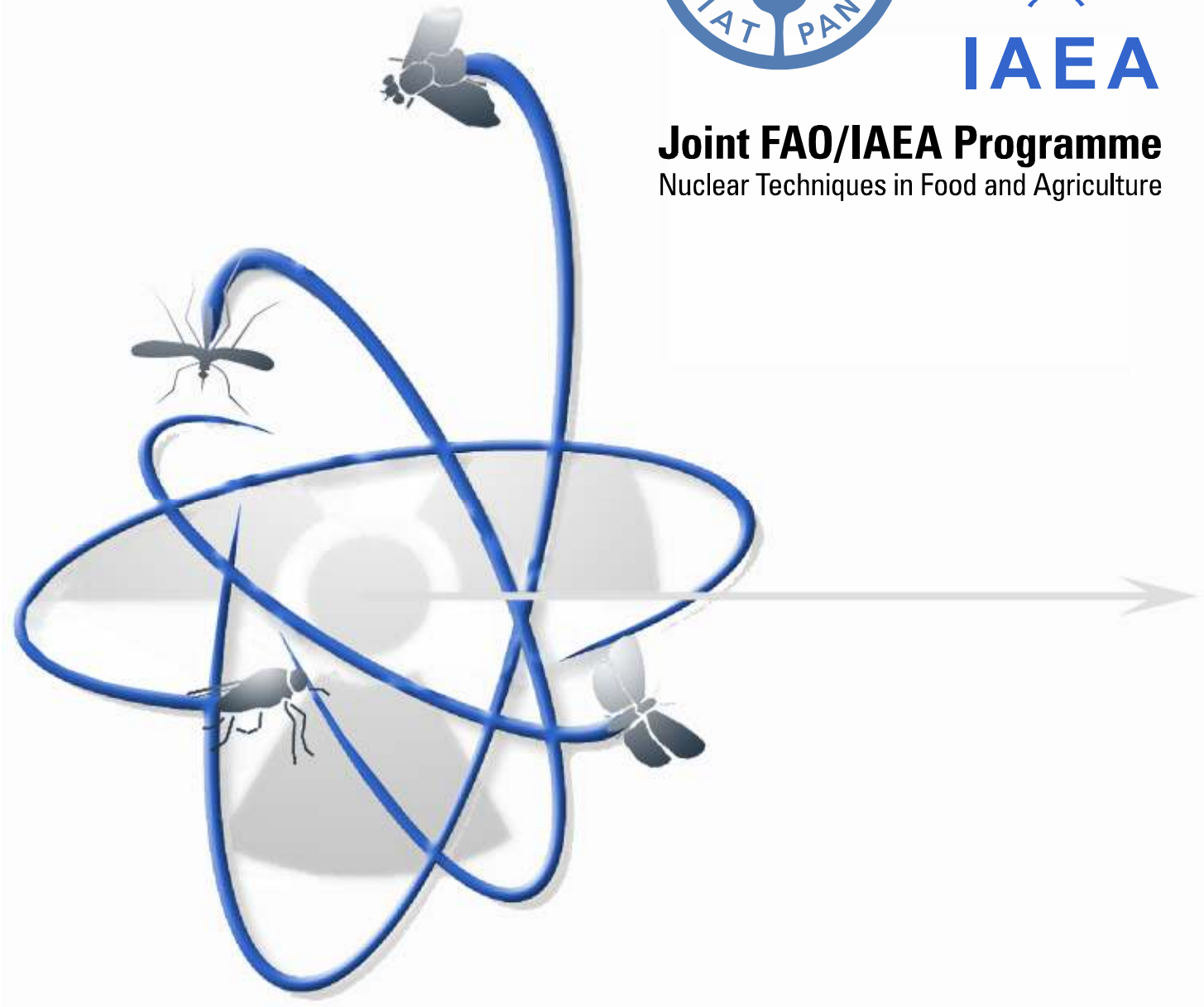

Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

ENTOMOLOGY UNIT

ANNUAL REPORT 2005

International Atomic Energy Agency
FAO/IAEA Agriculture and Biotechnology Laboratory
Agency's Laboratories, Seibersdorf

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1. Executive Summary

The use of the sterile insect technique (SIT) in area-wide integrated pest management (AW-IPM) programmes is becoming more and more part of mainstream insect pest control due to an expanding use of the technology and an improved regulatory framework. In March 2005 a new standard was adopted by the Interim Commission on Phytosanitary Measures which will greatly facilitate the export, shipment, import and release of sterile insects. This development will also provide a background against which commercialization of the technology can begin to expand. This new standard, along with others, which will probably be adopted this year, auger well for a strengthening of the area-wide concept in pest control.

The use of modern biotechnology plays an important role in several activities in the Unit, mostly in relation to the development of genetic sexing strains in mosquitoes and fruit flies. This type of technology causes concern in some areas and a position paper on this was developed in 2005 for presentation to the Departmental scientific advisory board. A biosafety committee was also established which followed the Austrian regulations and a member of the Unit is the secretary of this committee. Significant extra-budgetary funds have also been attracted to help fund this work.

The codling moth Cydia pomonella *is a major pest of pome fruits in very many parts of the world and there is a long-running SIT suppression programme for this pest being carried out in British Columbia. Several other countries are embarking on similar programmes and the question was raised as to whether there are any mating incompatibilities between codling moth populations from different parts of the world that would prevent sterile moths being reared in one facility and shipped for release elsewhere. This procedure is commonplace for fruit flies. Following receipt of diapausing larvae from many different populations worldwide an extensive series of field cage tests for mating compatibility was carried out and the results are presented in this report.*

Marking sterile insects so that they can be easily differentiated from wild insects is a very important tool in order to evaluate SIT programmes. This is traditionally done by fluorescent powder which does have some disadvantages. Very promising experiments have been carried out by using the stable isotope ^{13}C to label mosquitoes. Good retention of the marker was demonstrated and this work will be expanded in 2006. The costs of detection are currently quite high but new technologies are constantly reducing these costs.

In 2005, Mr Adly Abd Alla joined the Unit to carry out research on the tsetse virus and Ms. Sharon Soliban, Ms. Rebecca Hood-Novotny and Mr. Janis Thilayil were recruited to help support the mosquito activities. During 2005, three long standing staff of the tsetse group retired, Mr. Mamdouh Taher, Mr. Franz Ivanschitz and Ms. Gertie Gemershausen and Ms. Genevieve Labbe left the mosquito group to take up a position in the UK.

1.1 Tsetse Virus

The colony of *Glossina pallidipes* in the Unit has a long standing infection with a virus which causes salivary gland hypertrophy (SGH) in ca 4% of the individuals. These individuals are also sterile but as the SGH is at low frequency it does not compromise the ability to mass rear the strain. However, a newly collected field strain from Ethiopia that was established in the Unit eventually became extinct following two years of colonization and the frequency of SGH approached 100%. Considering the significance of the tsetse SIT project in Ethiopia research has begun to try to understand how the virus is transmitted in the colony in order to develop protocols to manage the infection. Ideally a virus free could be the solution but this may be difficult to establish and maintain virus free. Results using PCR have shown that although only ca 4% of the *G. pallidipes* colony show SGH, almost all are infected with the virus.

1.2 Marking Insects for Release

In the vast majority of SIT programmes insects need to be marked before release in order to be able to distinguish then from wild insects following their capture during the monitoring of the programme. Traditionally this is done using a fluorescent powder which adheres to the insect cuticle. There are several disadvantages to this method and in this report three alternative methods are described, two biological and one physical. In mosquitoes the stable isotope ^{13}C was used successfully to label male mosquitoes through adding ^{13}C to the water in which the larvae were reared. In fruit flies further work is reported on the using of a transgenic approach to insect marking as well as the incorporation of a phenotypic marker into a medfly genetic sexing strain.

1.3 Medfly Transgenesis

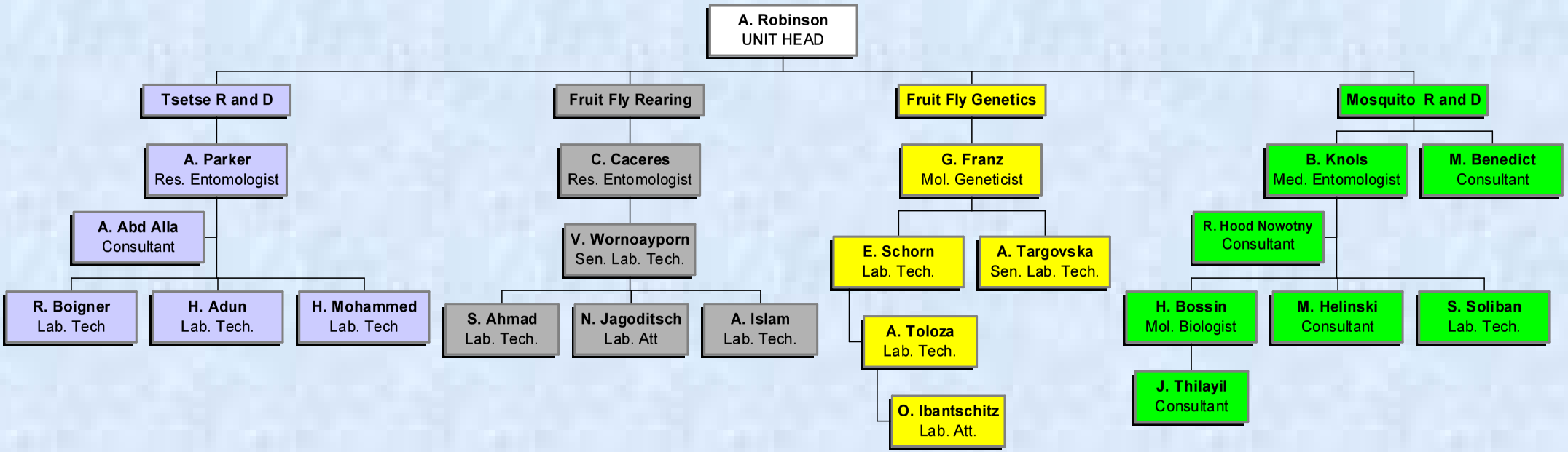
One of the key pre-requisites for the use of transgenic strains in any insect control programme is that the transgene should be 1) stable in the genome and 2) provide consistent expression of the effector gene. Extensive work has been carried out in the Unit to assess the veracity of these two pre-requisites in several medfly transgenic strains and some anomalies have now been identified which may indicate that not all transgenic strains meet these two criteria. The exact cause(s) of these anomalies is not known but they do suggest that more rigorous transformation systems may be needed. Taking this into account, new so called “suicide” vectors have been designed and during a consultancy by Dr. A. Handler from USDA, Gainesville, Florida, these vectors have been introduced into the germline of *Anastrepha ludens*. These new transgenic strains are now undergoing evaluation.

1.4 Sterility Induction in *Anopheles arabiensis*

In extensive study on radiation induced sterility in male *Anopheles arabiensis*, irradiated as either adults or pupae, has been carried out. Dose response curves for sterility were developed and the effect of radiation on egg hatch, survival and insemination was documented. Pupal irradiation was shown to lead to a small, but significant, reduction in the inseminating ability of the males, male survival did not seem to be affected by radiation of either stage. Based on the dose response curves for sterility and the other parameters, a radiation dose will be chosen in order to carry out competitiveness studies in large cages and eventually in semi-field settings.

ENTOMOLOGY UNIT ORGANIZATIONAL CHART

(as of December 31, 2005)

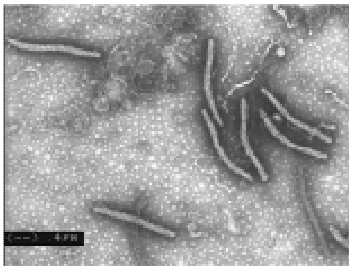


2. Tsetse R and D

With the gradual reduction of technical support for the tsetse activities in the Unit, decisions have been made concerning routine colony maintenance as well as outsourcing of some rearing components. Over the last years, with much support from the Unit, a tsetse rearing facility has been established at the Institute of Zoology, Slovak Academy of Sciences (IZ-SAS) in Bratislava. The facility now maintains a colony of ca 100,000 females comprised of three species.

The automated feeding and holding system for tsetse TPU3.2 was installed in the Unit in early 2005. This required considerable reconstruction and the provision of a new air-conditioning system. The unit will be able to hold a colony of 70,000 breeding females and an initial evaluation was carried out with G. pallidipes*. Some modifications were required following this evaluation and they have been carried out. The major modification involved ensuring that cages were aligned horizontally on the feeding membrane so that flies could obtain access to blood.*

Tsetse field populations from many species have been shown to carry a virus which leads, in a small number of cases, to salivary gland hyperplasia (SGH) and individuals with this symptom are also sterile. As reported in the ANNUAL REPORT 2003, a newly established colony of G. pallidipes *from Ethiopia, eventually became extinct probably due to this virus as the frequency of SGH reached almost 100%. Using virus specific PCR primers studies have been carried out in an attempt to learn more about the transmission of this virus under laboratory conditions and especially the role that membrane feeding may play in virus transmission. A non-destructive assay has been developed by which the presence or absence of virus in a fly can be determined by extracting DNA from an excised leg. The exact taxonomic status of the virus is unknown and it is planned to sequence the genome.*



Microbial decontamination of blood to be fed to tsetse is currently carried out using radiation. Whilst this is very effective it is not always possible to find a suitable radiation source with which to carry out this procedure and alternatives are being sought, one of them being UV irradiation. Following contact with colleagues in Scotland an initial trial was carried out with a UV irradiator to assess its effectiveness in reducing bacterial load in blood. Initial results were encouraging and the machine is now on loan in the Unit for further testing in 2006.

2.1 First Evaluation of TPU3.2

The development of a semi-automated system for tsetse holding and feeding has proceeded over a number of years in the Entomology Unit. Previous annual reports have included information on the earlier stages of the development. The design of the most recent version of the system, call the Tsetse Production Unit version 3.2 (TPU3.2), was completed in 2004. In order to test this version one half-length row of TPU3.2 was ordered for installation in the Entomology Unit. To accommodate the system, modifications were made to the building to make one long room from two former rooms. The TPU3.2 equipment was delivered at the beginning of 2005 and installed by March. Testing started from week 13 with *Glossina pallidipes*, with alternate units on the old trolley system and on the TPU3.2.

The initial results were very poor with high mortality in many of the units resulting in low overall fecundity. **Figure 1** shows the performance of the units under the two systems from unit 10 to unit 39 of 2005. Most of the units on the TPU3.2 have a value for pupae per initial female (PPIF) less than 2.3, the minimum for a self-sustaining colony.

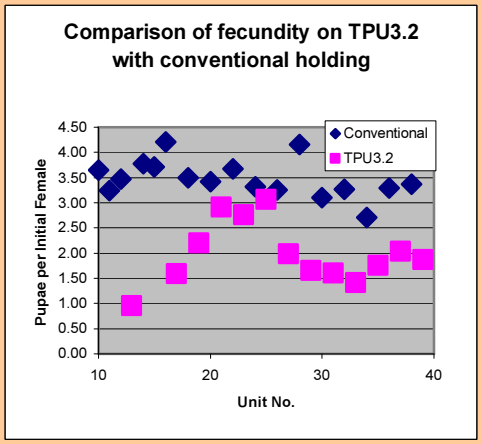


Figure 1. Fecundity, as pupae per initial female, for units held on the conventional trolley system or on the TPU3.2.

The principal problem was starvation, and this was attributed to problems with the alignment of the cages on the membrane system, and leveling of the cages. The alignment problems were addressed by adjustment of the TPU3.2, and it is planned to replace the runners on which the heating plates operate to increase their stability.

Cage leveling was a more difficult problem to address. On the TPU3.2, nine cages are held in a frame by tightening a clamp bar against the cages. The cages available in the Unit are made from 50mm sections of 200mm outside diameter grey PVC drainage pipe, covered on both sides with netting. The pipe wall thickness is about 4mm. These cages were made several years ago, and have been washed repeatedly at 60°C, and have had the netting replaced several times. During the spanning of the replacement netting, tension is applied to the cage, and when this tension is uneven the cages distort with the heat of the next wash. Over time almost all of the cages have distorted (**Figure 2**), resulting in difficulty with clamping the cages in the frame for the TPU3.2. In order to hold the cages in the frame it was necessary to tighten the clamp screws very tight, resulting in distortion of the frame and bowing of the cages. In addition adjacent cages did not sit snugly against each other, so that one cage would be lower then the next.

Figure 2. Old cage distorted by respanning netting and washing

To overcome these problems, the cage frame and cages were modified to positively locate the cages in a plane, and new cages obtained. The existing cage frame was easily modified by adding small locating pieces, consisting of 6mm rod mounted in a rectangular block that just fits inside the aluminium section of the cage frame, and by adding two aluminium rods across the width of the frame. The new cages (**Figure 3**) retain the original external dimensions, but incorporate several changes to the design. The new cages are made from a 6mm core foam PVC pipe instead of the original solid PVC. The foam core reduces the weight of the material, whilst the thicker wall increases stability and resistance to distortion. The netting is held by a galvanized metal spring set in a groove cut in the cage wall instead of by glue, allowing easier spanning of the netting and equalization of tension. Finally the cages have a 6mm diameter half-round groove cut in the centre line to engage with the cross rods and edge locating pieces (**Figure 3**). The new cage system is lighter and more ridged, and as there is no distortion to the frame it moves smoothly in the mounting blocks on the TPU3.2, so contacting evenly and firmly with the feeding membrane. The new cages and frames will be tested in early 2006.

Figure 3. New TPU3.2 cage showing net fastening and locating groove.

2.2 Collaboration with IZ-SAS

The progressive reduction of staff in the tsetse group over the last five years has reduced the rearing capacity in the Unit. In order to maintain our research and development role, a way was sought to transfer to another location the work of maintaining the larger colonies required to supply seed material to develop colonies in Africa. A survey of available options was conducted, and a request for tender developed. The main criteria identified for selecting a suitable location were cost, facilities, skilled staff and accessibility for technical support. Bids were received from five institutes, and from those the Institute of Zoology, Slovak Academy of Sciences, Bratislava (IZ-SAS) was selected as offering the best combination.

Figure 4. IZ-SAS insectary before and during conversion

Figure 5. Main holding room after completion

A contract was issued under the TC project INT/5/145 in December 2002, which continued until mid December 2005. Under the contract IZ-SAS had to develop, over three years, the facilities and staff to rear tsetse and to hold a colony in excess of 100 000 female flies. The IZ-SAS rented space from their sister institute, the Institute of Virology, which is adjacent to IZ-SAS. The rented space was the former

animal rooms, and required extensive modification (**Figure 4**). The external doors were closed up, the internal walls removed, and the floor relayed to make it level. The floors were tiled throughout and the wall tiling repaired. New equipment of the traditional design was supplied by a local firm. This consisted of the cages and holding trolleys, heating plates, feeding trays and membranes, and the emergence cages and chillers. Other standard equipment was supplied by the IAEA including air conditioners and humidifiers. After a delay of about five months caused by the late delivery of some of the equipment, the colony was initiated in September 2003 (**Figure 5**).

Initially a colony of *G. fuscipes fuscipes* was established, followed by *G. morsitans centralis* and *G. pallidipes*. Later the *G. m. centralis* colony was replaced by *G. m. morsitans*. By the end of 2005 the total colony size was 120 000 (**Figure 6**). As with the scaling up of any process, some difficulties were experienced as the colonies expanded. The greatest difficulty experienced, and still on-going, was with the *G. pallidipes* colony. This colony has shown itself to be unstable under rearing in many locations, including in the Entomology Unit. This instability is presumed to be due to the virus infection (see below).

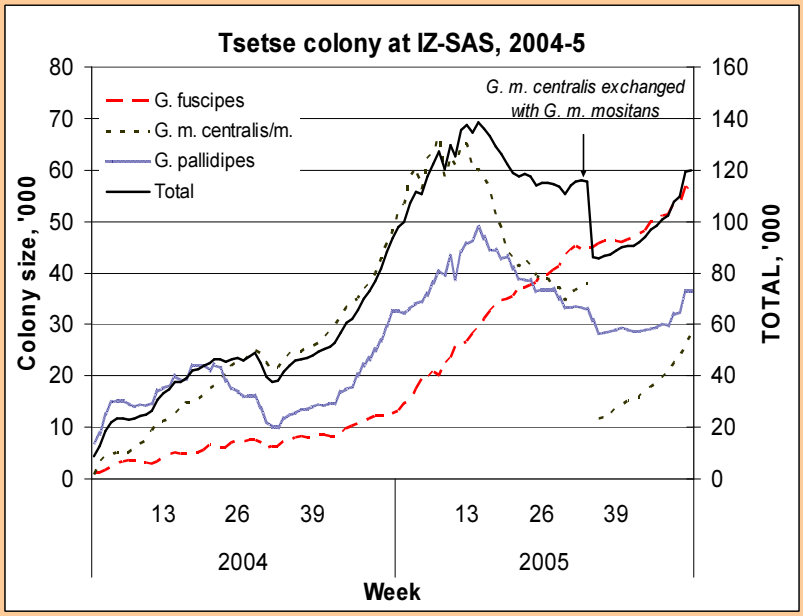


Figure 6. The growth of tsetse colonies at the IZ-SAS rearing facility

The main function envisaged for this facility is to provide material to the large insectary at Kaliti, Addis Ababa, Ethiopia. The Ethiopia project will require both *G. pallidipes* and *G. f. fuscipes,* both of which can be supplied by IZ-SAS. A new contract is being negotiated for this supply.

2.3 Current Status of the Tsetse Colonies

As a result of the loss of one staff position, it was necessary to reduce the colonies held in the Entomology Unit. The *G. pallidipes, G. brevipalpis* and *G. palpalis* colonies were maintained at their original level, but the *G. fuscipes* colony was terminated in week 29 once the colony in IZ-SAS was deemed to be large enough (see above). This IZ-SAS colony is continuing to grow and reached 55 629 at the end of the year.

A *G. morsitans centralis* colony had been initiated in IZ-SAS in 2004, and the remaining material from Seibersdorf was sent there in week 15 of 2005. However, the

demand for *G. morsitans morsitans* was greater than the Unit could supply, so it was decided to send the *G. m. morsitans* colony to IZ-SAS where it could be expanded and at the same time bring the *G. m. centralis* colony back from IZ-SAS to the Entomology Unit as there is currently little demand for this subspecies. This was done in week 37 and the *G. m. centralis* colony in the Entomology Unit stood at 4 849 and the *G. m. morsitans* colony in IZ-SAS at 27 947 at the end of the year.

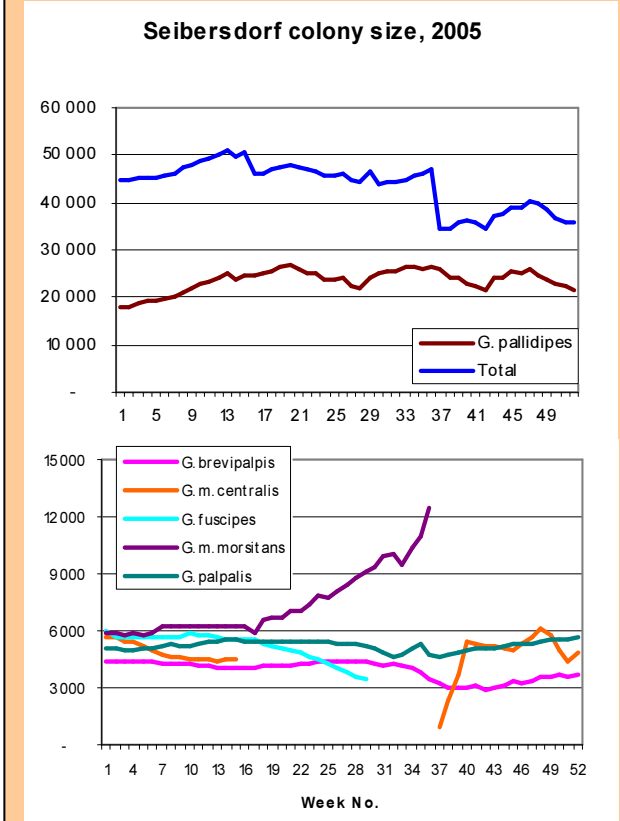


Figure 7. Tsetse colony status in the Unit throughout 2005

From the beginning of 2005, pupal shipments to TC projects were to be made against Requests for Procurement at the standard rate of US $0.88 per pupa. However no requests have been received. A few pupae were shipped at the beginning of the year to South Africa to complete a previous obligation. Shipment of pupae to research institutions has continued with reduced numbers according to the colony reductions noted above. The supply of *G. m. morsitans* and *G. fuscipes* was terminated and was taken on by IZ-SAS from mid 2005 as their colonies grew. Shipments for the year totaled 181 615, well below the approximately 500 000 pupae shipped in previous years because there was almost no shipment to TC projects.

2.4 Test of UV Treatment for Destroying Bacteria in Blood Diet

Tsetse flies feed only on vertebrate blood, which can be easily collected from a slaughter house. The flies though are very sensitive to bacterial contamination, which although it can be minimised by careful collection techniques and attention to hygiene is nonetheless unavoidable under the conditions of a slaughter house with contamination typically between 10-1000 cfu/ml. Up to now the blood has been treated with gamma radiation to reduce the bacterial load, 1 kGy producing approximately a 2 log reduction, but as the scale of rearing increases, the availability of large irradiators has become limiting. We have therefore sought alternative means of reducing the bacterial load in the blood. Pasteurisation and chemical treatment are both being investigated and have been reported in previous ANNUAL REPORTS.

Searching the literature, UV treatment is a relatively recent method used in treating blood products for human transfusion, principally to reduce virus contamination. UV treatment is also effective against bacterial, but it has not been tested in whole blood. We therefore decided to test UV treatment for bacterial reduction in whole blood.

The company IATROS in Dundee, Scotland, manufacture a system for blood component treatment which is principally used by the pharmaceutical industry. We have issued a contract to them to develop a system for our application. The contract is in three parts, and initial test of concept using a dummy medium, a second stage test with whole, defibrinated blood and indicator organisms (a bacterium and a bacteriophage) and a final stage of testing in Seibersdorf using normal tsetse diet to test the efficacy in bacterial reduction and quality and acceptability of the blood for tsetse feeding.

In the first stage we have developed in conjunction with IATROS a specification for a modified system to test with whole blood which IATRO assembled. Using a dummy feedstock and the test virus PhiX-174, a 4 log reduction of virus (LRV) was achieved with a residence time of 43 seconds (**Table 1**). The exposure was single pass, and the retention time was calculated based on flow rate and holding volume. Based on these results, a 4 log reduction could be expected with a throughput of one litre in 6.25 minutes. For the test with bacteria, a recirculating system was used with a large diameter irradiation pipe. A three log reduction was achieved in 10 litres of feedstock in 120 minutes.

Table 1. Reduction of the virus Phi-X 174 by UV irradiation of dummy feedstock

Retention time (s)	Log Phi-X 174	LRV
70	0	7.6
57	0	7.6
43	3.6	4.0
34	4.2	3.4
29	4.3	2.9
0	7.6	0

The second stage, also carried out by IATROS, involved the use of defibrinated, whole horse blood. For this test the recirculating system was again used. The blood was first adjusted to a standard haematocrit of 30% and a flow rate of 1500 ml min^{-1} was used, corresponding to a residence time of 4.56 sec per pass. For the bacterial test, *E. coli* was added to approximately 3 x 10^6 ml^{-1}, and for the virus test PhiX-174 was added to approximately 5 x 10^6 ml^{-1}. From each experiment, samples were taken at the start and each 15 minutes to 60 minutes for the viral test and to 120 minutes for the bacterial (**Figure 8**). For both tests complete elimination of the virus and bacteria was shown in 15 minutes, although some of the agar plates at the lowest dilutions still showed a few colonies. By 45 minutes significant production of methaemaglobin was detectable. Decontamination (minimum 3 log reduction) of a 20 litre batch of blood should be achieved in about 15 minutes.

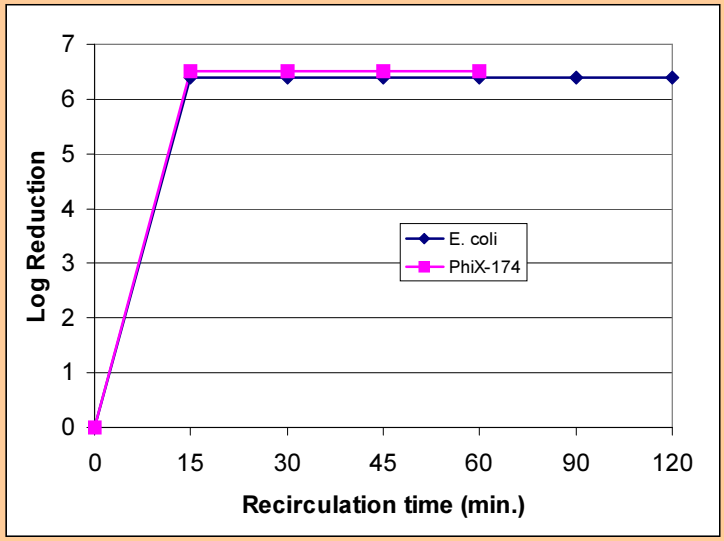


Figure 8. Bacterial and viral reduction in whole, defibrinated horse blood by UV irradiation.

Following the satisfactory result of the second phase, a UV irradiation system was shipped to Seibersdorf for testing during 2006. The principal issues to be addressed are the efficacy against the contaminant bacteria species encountered in our blood, reliability and ease of use of the system, and the effect of the irradiation on nutritional quality of the blood.

2.5 Salivary Gland Hypertrophy Virus (SGHV)

As reported earlier some tsetse species carry a virus that, in a certain proportion of individuals leads to salivary gland hyperplasia (SGH) and these individuals also show reproductive abnormalities. This virus is present in natural populations at a low level (0.5-5%), based on salivary gland dissection and in a colony of *G. pallidipes* maintained in the Unit, the frequency of SGH ranges form 4-10%. However, PCR analysis has confirmed that virus prevalence is 100%. The virus was also detected in samples of *G. pallidipes* from the colony maintained at the Kaliti facility in Ethiopia. Due to the negative impact of the virus on colony productivity under certain stressful conditions it is important to understand more about the virus with the goal to develop a management strategy for the virus. The most effective way to begin this study is to understand more about the virus in terms of its taxonomy and this can be done by obtaining nucleotide sequence information as recommended during a consultancy by Max Bergoin in 2004 (Montpellier). In July 2005, Drion Boucias (University of Florida, Gainesville) visited the Unit to develop collaborative work on this topic as he is working on a very similar virus in the house fly.

2.5.1. Production and purification of SGHV

To obtain the nucleotide sequence of this virus, sufficient quantities of viral nucleic acid are required and several methods were used to obtain the DNA. Firstly, hypertrophied glands were dissected from tsetse flies but due to the low percent of individuals with SGH (4-10%), a very large number of flies would need to be dissected. A second method tried was by artificial infection of larvae or adult flies. 3rd instar larvae were injected by virus leading to 90% SGH in surviving adults, however overall survival was very low (**Figure 9**) Adult flies were injected with virus and progeny will be screened for SGH. Hypertrophied salivary glands from dissected flies from the colony and those from the larval injections were used for virus purification. The virus was purified on a 20-60% linear sucrose gradient and confirmed using electron microscopy (**Figure 10**). Due to the long life span of tsetse, attempts to produce this virus in an alternative host are being under taken.

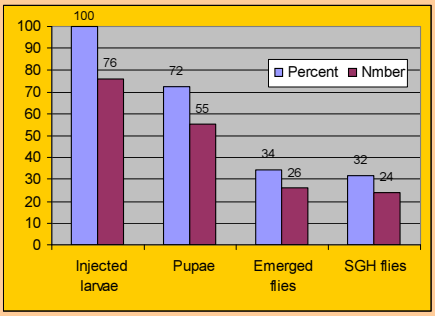


Figure 9. Producing SGHV through injection

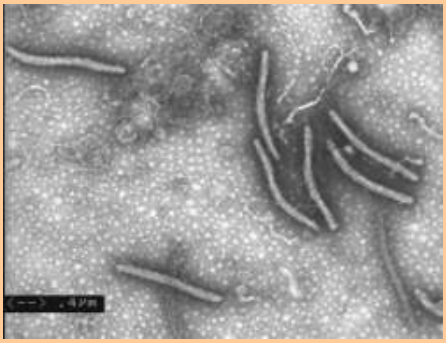


Figure 10. Purified SGHV under EM

2.5.2. Extraction of viral DNA, cloning and sequencing

Viral DNA was extracted using phenol-chloroform and the DNA precipitated with iso-propanol, centrifuged and resuspended in TE buffer. The quantity and quality

of DNA was checked using a spectrophotometer and by migration on agorose gel after digestion with *Eco*RI. The length of the DNA was estimated to be 185-220 kbp as previously observed by Max Bergoin (**Figure 11**). A small quantity of this DNA was used to construct two genome libraries. One using small inserts for easy cloning and sequencing and the second is a library of large inserts in order to facilitate sequence assembly. For the first library, the viral DNA was digested with *Eco*R1 for 2h at 37°C, de-activated by incubation at 65°C for 10min, then ligated with T4 DNA ligase in plasmid pUC19 previously digested with *Eco*RI, purified and dephosphorylated. Ligation products were transformed into TG1-competent bacteria by heat shock. Transformed bacteria were spread on LB agar plates containing ampicilin, X-gal and IPTG and incubated at 37ºC overnight. Mini-preparations of DNA from white colonies were carried out, and the length of cloned inserts was estimated by electrophoresis on a 1% agarose gel following *Eco*R1 digestion. Selected plasmids were sequenced using the M13 universal primer (MWG). We obtained more than 800 recombinant colonies and 681 colonies were analysed to estimate the insert length (**Figure 12**), and 415 inserts representing 60-90 kbp were sequenced. Attempts to assemble this sequence and to compare it with the data bank and to complete the genome sequence are ongoing.

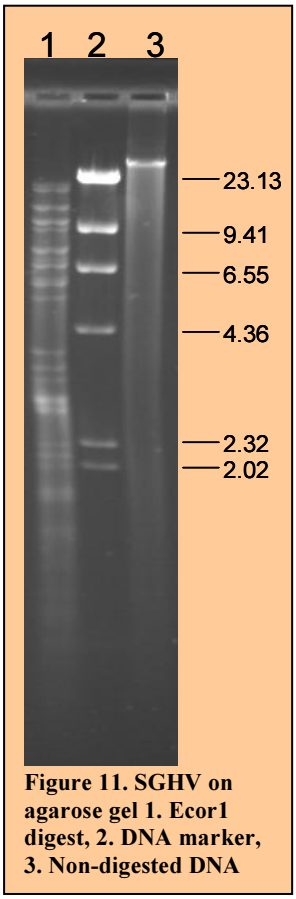


Figure 11. SGHV on agarose gel 1. Ecor1 digest, 2. DNA marker, 3. Non-digested DNA

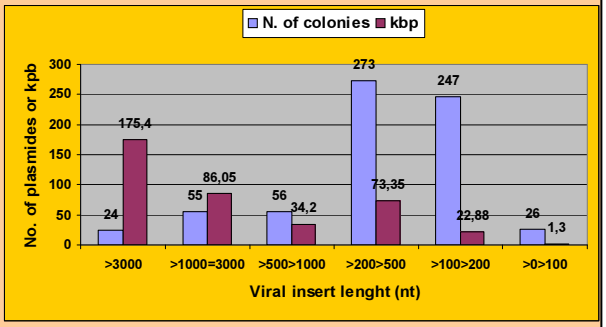


Figure 12. Recombinant plasmids and viral insert length

2.5.3 Mechanism of virus transmission in tsetse colonies

After the demonstration by PCR that virus is excreted into blood through feeding (ANNUAL REPORT 2004) it was important to assess if virus contaminated blood could be a source of horizontal transmission for the virus. Due to the absence of a strain without the virus this was difficult to demonstrate but feeding of flies on contaminated blood did not led to dramatic increase in SGH development. As vertical transmission of the virus was reported in the literature, an estimate of this was made in the *G. pallidipes* colony. Two hundred teneral flies were screened by PCR for virus prevalence with 17% being positive, however in flies over 80 days old 100% virus prevalence was recorded (**Figure 13**). Why does virus

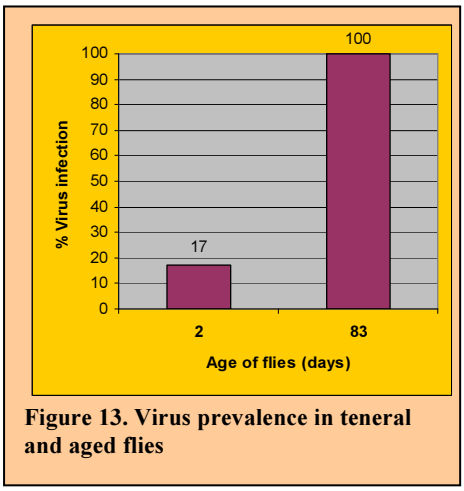


Figure 13. Virus prevalence in teneral and aged flies

prevalence increase with the age of the fly? Two hypotheses can be tested, the first is that the virus is present in all teneral flies but at an undetectable level and over time the virus replicates so as to be detectable in older flies. The second is that there is horizontal transmission of the virus through feeding or other contaminating means.

2.5.4 Screening for the virus in the G. pallidipes *colonies*

Using PCR, virus prevalence was assessed in the *G. pallidipes* colony and its relationship to fly age. As stated above there was a large difference between teneral and older flies in virus prevalence and **Figure 14** shows this relationship over time. It can be seen that after one week, virus prevalence jumps from 17% to 80% and thereafter remains very high. The reason for this rapid increase is not known.

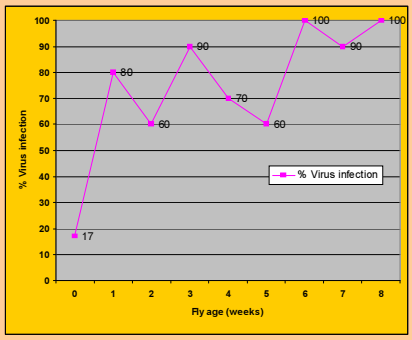


Figure 14. Virus prevalence and fly age

To assess the prevalence of SGH, more than 1500 flies were dissected and the SGH was recorded in female and male. The results show that 10% of the flies dissected had SGH with a higher percentage being recorded in males that females (**Table 2**). Most flies seem to be infected with the virus and yet show no symptoms. 10% infection is considerably higher than the 3% recorded in 2003. During these dissections it was observed that males with SGH can be easily identified even prior to dissection (**Figure 15**).

Table 2. Comparison of SGH in the *G. pallidipes* colony in 2003 and 2005

2003	Number of dissected flies	Number (%) with SGH
Female	1426	44 (3.1)
Male	585	32 (5.5)
Total	2011	76 (3.78)
2005		
Female	1147	120 (10.5)
Male	384	48 (12.5)
Total	1531	168 (11.0)

A **B**

Figure 15. Male tsetse, A: normal SG, B: male with SGH

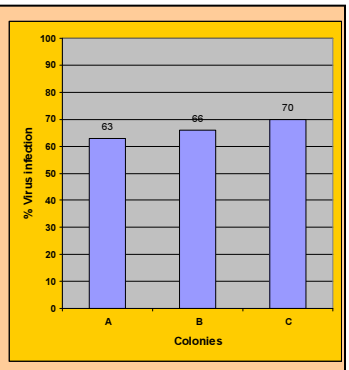


Figure 16. Virus prevalence in three *G. pallidipes* colonies from kaliti, Ethiopia

Attempts to establish a *G. pallidipes* colony in the Unit from material collected in the Southern Rift Valley, Ethiopia failed (ANNUAL REPORT 2000) probably due to very high level of SGH in the colony, which reached over 80%. Colleagues in the Kaliti facility have now been successful in establishing a colony but they are experiencing periodical disturbances in colony productivity. It was considered useful to assess the prevalence of the virus in this colony. A first assay on 90 flies from three colonies indicated prevalence, based on PCR, of ca 65% (**Figure 16**) and a much more thorough sampling of the colony based on 700 flies of different ages and from different sub-colonies showed virus prevalence rates of 80-100%.

2.5.5 Nucleotides sequence differences in SGHV from the Unit and Ethiopia

Viral PCR products from *G. pallidipes* originating from the Unit and Ethiopia were sequenced and compared by NTI vector 9 software. The alignment of the

sequences showed that all samples from the Unit were identical and all the samples from Ethiopia were also identical but different from those samples from the Unit. All sequences from Ethiopia have 4 nucleotides changes compared to the Unit sequence, i.e. A instead of G position 86, T instead of G position 107, T instead of C position 137 and A instead of G position 245. These are silent changes and do not affect the amino acid sequence. Using these sequence differences it was possible to design PCR primer sets that can differentiate between the two virus strains.

Using a mixture of three primers, the two original primers and the third being specific for the Unit flies the two virus strains can be unequivocally distinguished from each other. When the three primers are used on flies from the Unit two PCR products are produced of 401 bp and 290 bp but when flies from Ethiopia are used only one PCR product of 401 bp is produced (**Figure 17**).

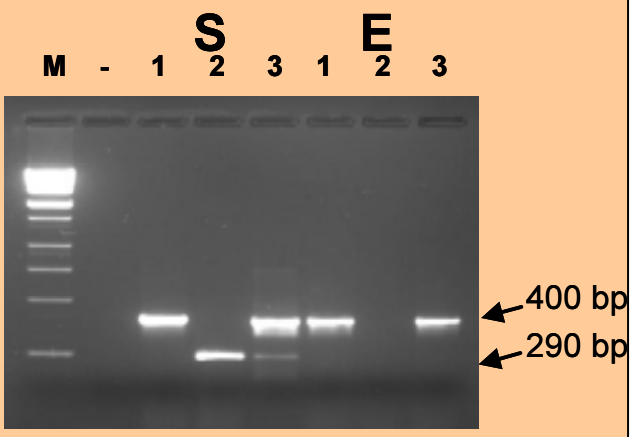


Figure 17. PCR products from virus strains from Seibersdorf (S) and Ethiopia (E). Seibersdorf specific 290bp band, M= marker DNA

2.5.6 Development of a non-destructive PCR method

In order to be able to select a virus free colony in tsetse a non-destructive PCR technique is required to assess the presence or absence of virus in individual flies, virus negative flies can then be used to initiate a colony. Initial trials showed that the virus could be detected in saliva collected from live flies and there was good correlation between detection of the virus from saliva and whole flies by PCR. The major disadvantage is that this method is laborious and cannot be used on a large scale. A second method involved PCR analysis of DNA extracted from an excised middle leg of a fly. Following optimization of PCR conditions there was good correlation between results from an excised leg and those from the whole fly (**Figure 18**). The method is easy and reliable and could be used to examine large numbers of flies to select negative flies for the establishment of a virus free colony.

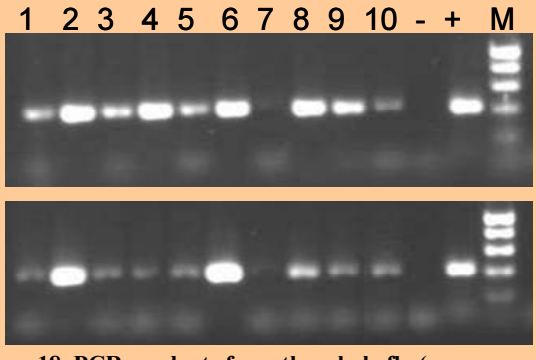


Figure 18. PCR products from the whole fly (upper panel) and from an excised leg (lower panel)

3. Genetic Sexing and DNA Analysis in Medfly

The use of transgenic techniques for the improvement of SIT requires that transgenic strains fulfill certain criteria and amongst these the most important are probably stability of the transgene in the genome and the stability of the expression of the transgene. In most laboratories carrying out this type of research, very limited numbers of insects have been studied in order to assess stability in these crucial areas. Data collected in the Unit with transgenic strains, evaluated at population levels comparable to those used to evaluate conventional genetic sexing strains, have indicated that the criteria of stability may not always be met. Although this is preliminary information it will be closely followed up as it is critical to any future use of this technology.

Effective marking strains of insects that have to be released in SIT programmes is an essential requirement in order that insects that are trapped in the field can be identified as sterile or wild. In most programmes this is done using a fluorescent powder which adheres to the adult insect. This procedure is expensive in terms of labour required to identify the insects and is not always foolproof. In this ANNUAL REPORT further information is provided on the use genetic and molecular markers to achieve the same end. The phenotypic mutant, Sergeant 2, is good example of a genetic marker as it is easily recognizable and does not appear to have any negative effects on competitiveness and it has now been incorporated into a genetic sexing strain. The other methodology described involves genetic transformation and the use of a transgene expressing a fluorescent protein.

Transformation technology has now also been transferred to another fruit fly species, Anastrepha ludens*, the Mexican fruit fly. This species is a major fruit pest in Mexico and there are SIT programmes being carried out for its control. In collaboration with colleagues in Mexico, a programme is underway to develop a genetic sexing strain for this species using both classical and molecular approaches. Initial transformation experiments have been very successful and strains are now available that carry transgenes that should confer increased stability. They are marked with two fluorescent proteins, red and green.*

3.1 Development of Marking Technologies for Released Sterile Flies

Flies released in a SIT programme are generally marked to allow discrimination of released and wild flies. Currently fruit fly pupae are dusted with a fluorescent powder that is picked up by the flies during emergence. This technique is rather costly (materials and labour) and is not entirely accurate and two genetic/molecular strategies can be considered: a) the use of a particular mitochondrial DNA haplotype that is different from most wild type populations (ANNUAL REPORT 2004). The drawback of this methodology is that this characteristic is not phenotypically visible, i.e. the fly DNA has to be isolated first followed by a PCR analysis. Furthermore, an enormous knowledge about the polymorphisms present in the different wild populations is required and b) phenotypic marking of the flies either with a classical mutation (e.g. *Sergeant 2*, Sr^2; discussed below) or through transgenesis using genes expressing a protein that fluoresces under appropriate light conditions.

3.1.1 Mapping of the mutation on chromosome 5

The mutation *Sergeant 2* (Sr^2) was discovered by A. Zacharopoulou (University of Patras). It shows three white stripes on the abdomen (**Figure 19**). Genetic analyses showed that Sr^2 is a dominant lethal mutation that is located on the right arm of chromosome 5. To determine the exact position a deletion experiment was performed. Pupae were irradiated either with 40 or 50 Gy one day before emergence. To be able to distinguish Sr^2 deletions from the respective wild type a special strategy was required. The irradiated males with a Y-autosome translocation (CC59) that carried Sr^2 were crossed with *w wp* Sr^{2+} females. The progeny were screened for exceptional males with a w^+ wp^+ Sr^{2+} phenotype. Among ca 6400 flies screened two such flies were detected (**Table 3**) and polytene chromosomes were prepared from salivary glands and male sub-orbital frontal bristle (SOB) trichogen cells (A. Zacharopoulou) and Sr^2 was located at 76A and 60D, respectively (**Figure 20**), i.e. very close to *wp* and even closer to *tsl*.

Figure 19. Wild type (upper) and Sr^2 (lower)

Table 3. Progeny of Irradiation of T(Y;5)CC59 w^+ wp^+ Sr^2/*w wp* Sr^{2+} males crossed with *w wp* Sr^{2+} females

Dose	w^+ wp^+ Sr^2 males	*w wp* Sr^{2+} females	w^+ wp^+ Sr^{2+} males	Total
40 Gy	2207	1735	0	3942
50 Gy	1436	1032	2	2470

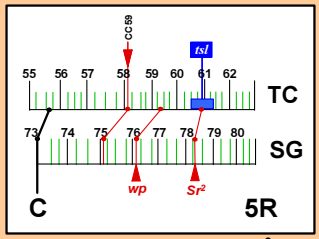


Figure 20. Mapping of Sr^2 on salivary gland (SG) and trichogen cell (TC) polytene chromosomes

Sr^2 was also mapped genetically in relation to some of the other mutations on chromosome 5. Male and female recombination frequencies were determined in both reciprocal crosses. In addition the recombination frequency was measured in males carrying the Y-autosome translocation T(Y;5)101. Furthermore, in most cases the recombination frequency was also determined in the presence of the inversion D53. Large numbers of individuals were used to obtain reliable results

especially when the male recombination frequency was determined. **Figure 21** shows the results and they can be summarized as follows.

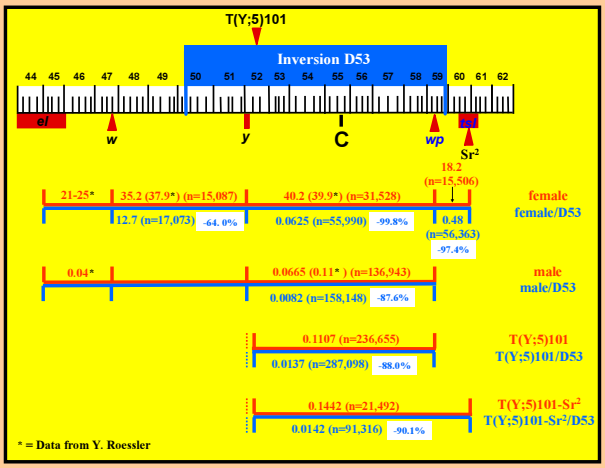


Figure 21. Genetic map of the mutations *elbowed saetae* (*el*), *white* (*w*), *yellow body* (*y*), *white pupae* (*wp*), *temperature sensitive lethal* (*tsl*) and *Sergeant 2* (*Sr²*). A schematic drawing of the polytene chromosomes from SOB trichogen cells is shown. (C = centromere)

Wherever comparable results are available the results agree very well with those published by Y. Roessler. The genetic distances between the markers used are generally in agreement with their physical distance on polytene chromosomes. As expected the recombination frequency in males is significantly lower than in females. The presence of the inversion D53 reduces the frequency of recombinants significantly; however not to zero. The reduction of detectable recombinants resulting from exchange in the *y-wp* interval (completely covered by the inversion) ranges from -87.6 to -99.8 %.

The presence of the inversion has also a significant effect on the recombination in the neighbouring regions *w-y* and *wp-Sr²*, i.e. regions that are only partially covered by inversion D53. For the *wp-Sr²* interval the reduction in recombinants (-97.4%) is equal to that found within D53 although only a relatively small fraction (ca 25%) is included in the inversion. In case of *w-y* the reduction is less sever (-64%) despite the fact that roughly half of this interval is covered.

Chromosome inversions do not reduce the recombination frequency as such. Fewer recombinants are detected in the offspring of parents heterozygous for an inversion because any uneven number of recombination events within the inversion leads to recombinant chromosomes with deletions and duplication. The resulting genetic imbalance in the offspring carrying such recombinant chromosomes leads to lethality and therefore to an overall reduction in recombinants.

3.1.2 Construction of a GSS where the males carry Sr²

Because *Sr²* is a dominant lethal the only possible way of incorporating this mutation into a GSS is to link it to the Y chromosome. Under normal circumstances one would have to induce new translocation by irradiating *Sr²* males (as it was done for CC59). However, we took advantage of the fact that the Y-autosome translocation breakpoint in the current GSS (T(Y;5)101 = VIENNA 8) is sufficiently far away from *Sr²* allowing the mutation to be introduced into the translocation via male recombination. For this T(Y;5)101 males were crossed with heterozygous *Sr²* females and the F1 males with *Sr²* phenotype were crossed with *w wp* females. The resulting F2 was screened for exceptional *Sr²* males. Among ca 21000 flies screened 18 such males were detected. One of these males was used to construct a GSS by backcrossing twice with D53 females.

3.1.3 Stability of a GSS carrying Sr^2

The rearing of the Sr^2-labeled GSS was brought to the standard level (per generation 34 ml of pupae are used to set up the next generation and a parallel sample of 40 ml is analysed for possible recombinant flies). So far the strain has been reared in this manner for 40 generations and ca 91000 flies have been screened. Among these four wp^+ Sr^2 females (= 0.0044%) and 6 wp Sr^{2+} males (= 0.0066%) were detected. These are the reciprocal phenotypes that would be expected if recombination takes place in the chromosomal region between the translocation breakpoint and *wp*. One exceptional male showed a wp^+ Sr^{2+} phenotype. This could be the consequence of a recombination event between *wp* and Sr^2. An alternative explanation for this single male would be that the Sr^2 mutation is not 100% penetrant. In addition, three wp^+ Sr^{2+} females were found. They could be explained by a double recombination, one in the region between the breakpoint and *wp* and a second one between *wp* and Sr^2. The overall recombination frequency between the translocation breakpoint and *wp* is 0.0142%, i.e. very similar to the 0.0137% as measured for the same strain without Sr^2. The latter strain was reared for 81 generations and over 195000 flies were screened during that time. Recombination between *wp* and Sr^2 occurs with a frequency of 0.0044%.

As mentioned above Sr^2 is located very close to the *tsl*. This makes the mutation a valuable marker for the detection of recombination in the *wp-tsl* interval. In the filter rearing system, GSS are cleaned based on the pupal phenotype, i.e. recombinants where the recombination has occurred between the translocation breakpoint and *wp* can be detected and removed. However, a recombination between *wp* and *tsl* can only be detected via a temperature test. In a strain with Sr^2 such recombinants are visible.

The number of adults per 40ml is comparable to that of a strain without Sr^2 (**Table 4**), i.e. the addition of Sr^2 does not seem to have a major negative impact on these QC parameters. The productivity of a strain can also be evaluated using the data obtained from the temperature tests. At regular intervals the strains are subjected to this test to determine whether they break down with respect to the *tsl* mutation. **Figure 22** summarizes the results for 3 strains: T(Y;5)101-Sr^2 with *w wp* or with D53 and T(Y;5)101 with D53. The strains with D53 show the expected decline in hatch with increasing temperatures. Accordingly the number of white pupae and females falls of. The number of brown pupae and males stays more or less constant throughout the entire temperature range. At 34˚C, the temperature normally used in the sexing procedure, T(Y;5)101-Sr^2/D53 produces 248 male adults as compared to 278 and 279 in the other 2 strains.

Table 4. Productivity of GSS T(Y;5)101/D53, with and without Sr^2

	T(Y;5)101/D53	T(Y;5)101-Sr^2/D53
Total males	1426	1394
Total females	1051	1143
Good males	1343	1301
Good females	974	1046

Overall it can be concluded that the Sr^2-labelled strain performs well, at least for the characteristics analysed here. Additional experiments were performed to evaluate the mating performance of the strain (ANNUAL REPORT 2000). Also here

the results were positive. However, the major drawback of the strain is that only the males are labelled.

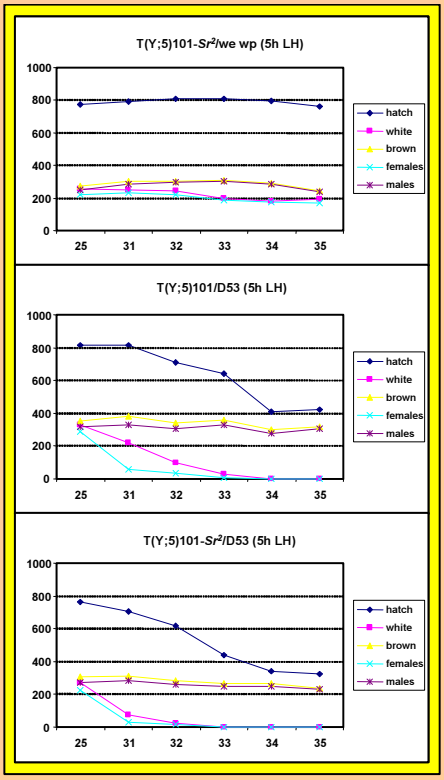


Figure 22. Temperature tests with 3 different strains. 5h LH = eggs were collected for 5 h, treated for 24 h with 25˚C (L) followed by a 24 h treatment with temperatures between 31˚C and 35˚C (H). The results are presented for 1000 eggs per treatment.

3.2 Further Analyses of Transgenic Medfly Strains.

One of the primary reasons for the use of transgenesis for SIT programmes is the construction of strains that carry an easily identifiable marker that allows discrimination of released and wild insects. Good candidates for such a marker are the various fluorescent proteins currently available, e.g. EGFP and DsRed. One principle prerequisite for such marker to function in the field is that the respective protein survives long enough in dead flies. That is necessary because the monitoring in the field is done with traps that are usually serviced once a week. For EGFP-labelled strains we have shown that the viability of the fluorescent protein in dead flies is very high (ANNUAL REPORT 2002), i.e. in some cases, and depending on the overall strength of the initial fluorescence, the marker is visible even months after the death of the flies. Furthermore it was noted that four different patterns of expressions, and within each pattern different levels of intensity, can be observed although in all transgenic lines the same promoter was used to express the marker (ANNUAL REPORT 2002). However, DsRed is probably the better marker for a practical application because the observed fluorescence is much stronger than that of EGFP.

In collaboration with A. Handler (USDA Gainesville) we constructed several strains that carry DsRed under the control of the polyubiquitin promoter. The aim is to incorporate the marker into a classical sexing strain. Such a strain contains the inversion D53 carrying the markers *white pupae* (*wp*) and *temperature sensitive lethal* (*tsl*) and a translocation linking the wild type alleles of the markers to the male-determining Y chromosome. The females in such a strain are homozygous and the males heterozygous for the markers. The easiest way to include the DsRed marker would be if the insertion site is on the D53 chromosome. To identify insertions on D53 all transgenics made with this strain were analysed

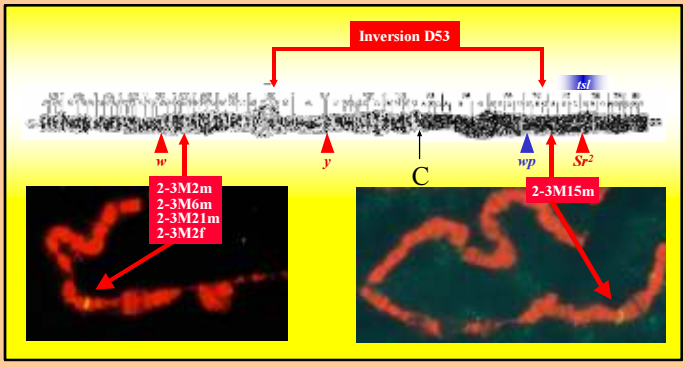


Figure 23. Revised map of chromosome 5 showing inversion and the insertion sites

genetically. The different families heterozygous for the transgene were crossed with the wild type strain EgII. The F1 was inbred and the F2 was screened for families where the fluorescence and *wp* co-segregate (flies with *wp* phenotype were used to set up homozygous lines). Some of the families where this is the case were also analysed cytologically using *in situ* hybridisation on polytene chromosomes from salivary glands (collaboration with A. Zacharopoulou, University of Patras). **Figure 23** shows a revised map (as compared to the map in ANNUAL REPORT 2002) of chromosome 5 and the positions of five transgene insertions. The cytological data show that we have several strains, originating from the same G1 male (2-3M), with either an insertion at 66B or at 76A. Three of these strains were used for further experiments, i.e. 2-3M2m, 2-M2f and 2-3M15m. All strains show a relatively low level of fluorescence, especially 2-3M15m. This becomes more apparent when the insertion is present only in a heterozygous condition (**Figure 24**). This is clearly a drawback for the use in a sexing strain as the males carry only a single copy of the marker gene.

Figure 24. Medflies heterozygous and homozygous for insertion 2-3M2m

One homozygous strain, 2-3M2m, was used to construct a sexing strain by crossing females to males carrying the translocation T(Y;5)101. The F2 males were backcrossed with the respective females and the resulting sexing strain was maintained by inbreeding. Throughout the construction of the sexing strain the fluorescence was checked and only flies with the correct fluorescence were used. The rearing level was increased to our standard level, i.e. each generation 34 ml of pupae are used to set up the next generation and a parallel sample of 40 ml of pupae was analysed.

Table 5 shows the results of several generations of inbreeding. A relatively high percentage of non-fluorescing flies, especially in the males, was detected. As a consequence the original homozygous strains were re-examined to see whether they also show the same phenomenon. If one counts sufficiently high numbers of flies also here a certain proportion of flies can be detected that shows no fluorescence. In this context the following three observations were made.

Table 5. VIENNA 8 DsRed with 2-3M2m

	Pupal phenotype (#)				Fluorescence phenotype (%)			
	Females		Males		Females		Males	
Gen.	wp^+	*wp*	wp^+	*wp*	weak	"-ve"	weak	"-ve"
10	0	790	1100	0	1.9	0.89	6.4	10.18
11	0	597	1476	0	2.18	1.01	7.32	9.28
12	0	979	1294	0	0.31	0	3.79	6.57
13	0	769	1257	0	1.43	0.13	3.02	10.42
14								
15	0	1100	1561	0	0	0	3.01	9.99
16	0	890	1451	0	0.56	0	3.85	4.4
17	0	799	1403	0	0.13	0	6.56	2
18	0	826	1209	0	0.61	0	3.8	6.2
Total	0	6750	10764	0	0.79	0.21	4.71	7.33

The classification "-ve" was later (after aging the flies) changed to extremely weak.

1) Crossing of the non-fluorescing males from the sexing strain with EgII females leads to offspring where most of the females show very low and the males no fluorescence (**Table 6**). The result that all females and none of the males show fluorescence is explained by the fact that the parental males carried a Y-autosome translocation without a DsRed insertion.

2) It was attempted to select the three homozygous DsRed lines and a parallel line of the sexing strain for maximum expression of the fluorescence. For over 20 generations the flies with highest fluorescence were chosen to set up the next generation. This procedure did not lead to a homogeneously high level of fluorescence in the strains, i.e. still some weak expressing flies are detected each generation.

Table 6. Result of test crosses with "negative" males from VIENNA 8 DsRed with 2-3M2m

Fluorescence	Male 1		Male 2		Male 3	
	Males	Females	Males	Females	Males	Females
negative	43	4	127	3	69	4
very weak		17		44		31
weak		6		12		0
strong		1		24		12
	Male 4		**Male 5**		**Total**	
	Males	Females	Males	Females	Males	Females
negative	116	5	108	2	463	18
very weak		73		83		248
weak		31		18		67
strong		0		0		37

3) Later it was observed that aging of the flies (for 5-7 days) leads to an increase in fluorescence in the initially negative individuals. During the routine inbreeding (e.g. for the data in **Table 6**), the flies were screened shortly after emergence. However, even after aging the flies the fluorescence is extremely low and can probably only be detected by very experienced persons.

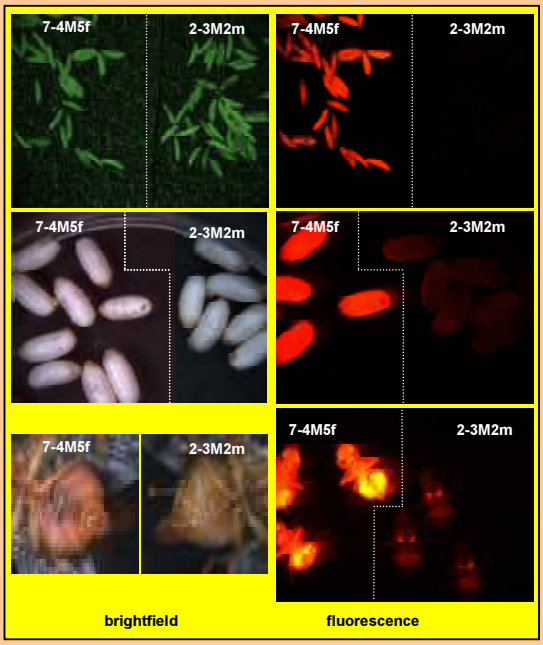


Figure 25. Comparison of fluorescence in two medfly strains

It can therefore be concluded that the variation in the fluorescence is not due to a loss of the transgene but is caused by extremely low levels of expression in a certain proportion of the flies. For a mass rearing experiment, planned as next step of the evaluation of the DsRed-labelled sexing strain, where large numbers of flies have to be screened every generation this situation is not ideal.

We therefore selected another DsRed-labelled strain where the insertion is on D53 (7-4M5f). As compared to all other strains that we have examined so far this strain shows a remarkable intensity of fluorescence at all developmental stages (**Figure 25**). Furthermore, the fluorescence is even visible without a fluorescence lamp, i.e. pupae and adults show a pink colour. Despite the very high level of fluorescence the homozygous and heterozygous condition are distinguishable.

The fluorescence in the thorax is weaker in heterozygous flies. The fluorescence observed in freshly laid eggs is the result of maternal material. If a 7-4M5f male is crossed to an EgII female no fluorescence is observed in young eggs. The fluorescence appears 2 days after egg deposition, i.e. 8 hours before hatch.

Initially this chromosome was maintained heterozygous by crossing each generation of fluorescent flies with D53 flies. The DsRed-labelled chromosome was made homozygous by crossing 7-4M5f flies either with EgII or with two balancer chromosomes. The results of these crosses are shown in **Table 7**. In case of the crosses with the balancers some exceptional phenotypes were observed, i.e. wp^+ and $wp\ Sr^+$ flies without fluorescence. The $wp\ Sr^+$ flies with fluorescence from the cross with balancer 68 *y Sr* were selected as a homozygous strain.

Table 7. F2 of the crosses to generate the homozygous 7-4M5f strain

Strain	wp^+ DsRed	wp^+ -ve	$wp\ Sr^+$ DsRed	$wp\ Sr^+$ -ve
Balancer 68 *y Sr*	84	10	39	0
Balancer 68 *Sr*	72	6	51	2
	wp^+ DsRed	wp^+ -ve	*wp* DsRed	*wp* -ve
EgII	143	63	40	0

Through the standard crosses with males carrying the translocation T(Y;5)101 a sexing strain was generated. Currently this strain is in generation 3 of the standard rearing procedure. The results obtained so far are shown in **Table 8**. In the second generation of the crosses to construct the strain as well as in the first two generations of the standard rearing procedure males without fluorescence were detected with an average frequency of 3.54 %. Five of these males were crossed with EgII females. In the offspring no fluorescent flies were detected. In this case also ageing of the flies did not change the phenotype. Further molecular and cytological analyses will be required to determine whether these exceptional flies have lost the transgene or whether this represents an extreme case of low expression. Furthermore, the crosses are now being repeated to generate a homozygous 7-4M5f strain. In contrast to the previous experiment single pair crosses will be used to eliminate any heterogeneity that might exist in the heterozygous strain.

Table 8. VIENNA 8 DsRed with 7-4M5f

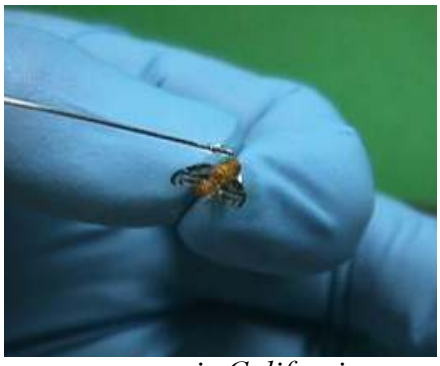
	Pupal phenotype (#)				Fluorescence phenotype (%)			
	Females		Males		Females		Males	
Gen.	wp^+	*wp*	wp^+	*wp*	**weak**	**-ve**	**weak**	**-ve**
F2	0	524	852	0	0	0	0	1.64
2	0	906	1213	0	0.11	0	0.25	1.98
3	0	908	991	0	0	0	0.5	5.45
Total	0	1814	2204	0	0.06	0	0.36	3.54

F2 = second generation of the backcrosses to create the GSS (less than 40 ml were screened). This generation was not included in the total. In the females fraction individuals are detected where the thorax is less fluorescent.

4. Fruit Fly Rearing and Quality Control

Developing improved diets, either in terms of cost or rearing efficiency, for potential targets for SIT is an important part of the work of this group. Currently two species, Bactrocera oleae *and* Anastrepha fraterculus *are investigation, the former in terms of reducing cost and the latter in terms of improving rearing efficiency. Studies in the Unit have already identified a much cheaper replacement for the cellulose as bulking agent for the larval diet of* B. oleae*. The success in rearing this species to quite large numbers has been recognized in Member States as many requests are now being received to supply pupae for this species. Further work reported here indicates that replacements may also have been found for another expensive larval diet component i.e. soya hydrolysate. For* A. fraterculus *attention has focused on identifying an improved adult diet which increases egg production and egg viability*

Treating some species of fruit flies with precursors of the male pheromone or analogues of juvenile hormone can significantly accelerate sexual development in males. This can be of great practical significance as it would enable sterile males to be released as more competitive insects. During two consultancies in 2005, experiments were carried out to see if this observation could be extended to new species and strains. In medfly, this type of technology has been introduced into the preventative SIT release programme in California.



In the 1970's there was much interest in developing the SIT for the olive fly, B. oleae*. Two major problems were identified, namely the cost of production (but see above) and the low competitiveness of the sterile males. This low competitiveness was traced to a difference in daily sexual activity of the laboratory males in comparison with the wild males. In this report initial studies of this phenomenon for several different strains are reported. This work will be continued with the implementation of field cage tests using wild olive flies.*

4.1 Rearing the South American Fruit Fly *(Anastrepha fraterculus)*

South American fruit fly *Anastrepha fraterculus* in contrast to most of the other fruit flies does not adapt well mass rearing in relation to egg viability when the adults are fed with the fruit fly diet based on sugar and yeast hydrolysate (3:1). To overcome this problem several products, such as corn extract protein have been used as an additional supplement to improve egg viability for A. *fraterculus* in other facilities (e.g. Argentina and Brazil). However corn protein extract is not a standard industrial product and preliminary experiments have been conducted to evaluate the possibility to use wheat germ as a supplement for the feeding adult *A. fraterculus.*

The "germ" is the most nutritious portion of the wheat kernel and makes up only about 2.5% of its weight. Wheat germ is the reproductive area or embryo from which the seed germinates. Wheat germ is usually separated from the bran and starch during the milling of flour because the germ's perishable oil content limits the keeping time of the flour. Wheat germ is packed with protein, fiber, polyunsaturated fat, vitamins (e.g. B-complex vitamins), and minerals. Wheat germ is abundantly rich in protein (28.9%). High quality protein is absolutely essential to allow females to produce good quality and quantity of eggs.

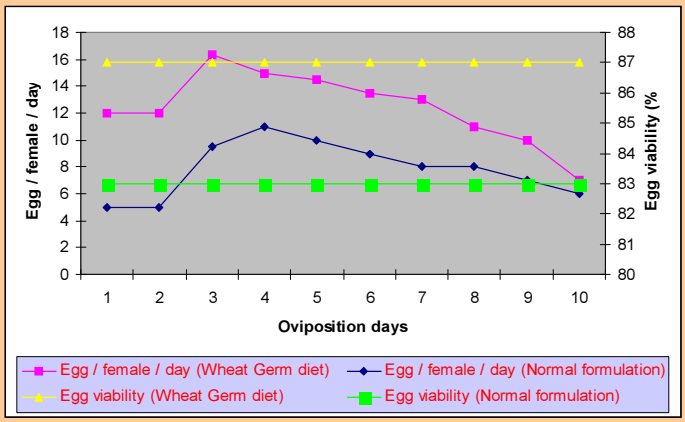


Figure 26. Fecundity and egg viability in *A. fraterculus* females fed different diets

Results (**Figure 26)** indicated that the diet formulation of 3:1:1 (sugar, yeast hydrolysed and wheat germ, respectively) can increase the egg production per female from ca 9 to ca 16 eggs per female per day and hatch from 83%±0.03 to 87%±0.02 when compared with standard fruit fly adult diet 3:1 (sugar and protein respectively). Additional experiments are on ongoing to determine optimal diet formulation to measure the effect on egg production and subsequent offspring quality.

4.2 Olive Fly, *Bactrocera oleae*

4.2.1 Mass rearing

As reported earlier (ANNUAL REPORT 2004) the expensive cellulose bulking agent in the larval diet has been replaced with an inexpensive type, which is derived from the industrial paper production. This will enable olive flies to be produced at a much reduced cost for any future SIT project. However, in the current larval diet formulation there are still some expensive components such as soya hydrolyzate which could be substituted by other inexpensive ingredients with similar nutritional proprieties. Currently experiments are underway to assess if soya flour and/or wheat germ can replace the soya hydrolyzate and initial results are very promising.

4.2.2 Sexual activity of different olive fly strains

Three colonies of olive fly are maintained in the Unit. One from Democritus, Greece (more that 30 years old), a hybrid strain (5 backcrosses between Democritus ♀ x Valencia ♂ one year old) and a new laboratory colony received from Dr. Mark

Robertson USDA-ARS, California. This strain was colonized with wild material from infested olives collected in California. These strains have been maintained in order to run strain behaviour and compatibility studies.

In collaboration with a consultant from University of Heraklion, Greece, Mr. Antonis Chrisantis, field cage tests were conducted to determine the sexual activity profile of three strains, a laboratory colony from Heraklion, Greece; the Seibersdorf colony (originally from Democritos Greece) and the laboratory strain from California. In this trial 100 males and 100 females of same strain were released in the field cage, around 9.00a.m. In all tests there was very little fly movement for the first 2h with most flies being found on the cage netting in groups of 20-30. After this period the flies started to approach the host trees and the first mating started at about 13.00–14.00 when light intensity was 3050 lux. The sexual activity was maintained until sun set with most matings occurring when light intensity was between 400 to 2 lux (**Figure 27**). For flies from Heraklion, the first mating was observed at about 13.00–14.00 when light intensity was 1450 lux and the peak of sexual activity for this strain occurred when light intensity was 100-200 lux. The flies from California only started mating at about 15.30 (187 lux). One possible explanation for this is that the Californian colony is a new colony (about 4-5 years old) and may still have some behavioral elements of the wild population, which normally starts mating at the end of photophase, slightly before the sunset. It was also concluded that laboratory flies from all colonies had a good index of participation in mating. Further experiments will include wild insects to measure sexual compatibility between sterile laboratory flies from these different strains and wild insects.

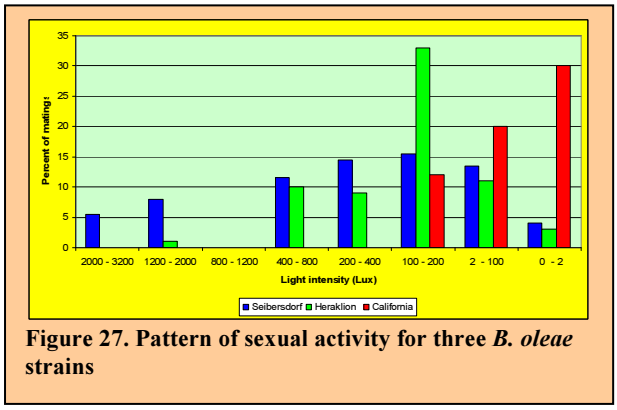


Figure 27. Pattern of sexual activity for three *B. oleae* strains

4.3 Hormonal Treatments for *A. fraterculus, C. capitata* and *B. dorsalis* Males

Previous studies conducted with *A. ludens*; *A. suspensa* and *C. capitata* have shown that either topical application or feeding juvenile hormone during the early adult stage can speed up sexual maturity and increase sexual activity of sterile males. Use of this therapy in SIT programmes could greatly increase efficiency and would represent considerable savings in space and energy that is normally required to allow the males to become sexually mature. During a consultancy by Dr. Peter Teal from USDA-ARS Gainesville, studies on this hormone therapy were conducted on another fruit fly, *A. fraterculus* as well as on a medfly genetic sexing strain.

4.3.1 Treatment of males of the medfly GSS Vienna-8

Topic application of methoprene or acetone as control was applied to males on the day of emergence and small cage mating tests using 2-day old males and mature females were conducted. However, males of this strain reared in the Unit mature very much faster than those of the VIENNA 7 strain reared in El Pino, Guatemala and supplied to Dr. Teal's laboratory in Florida. The latter males require 5-6 days to become fully mature and a clear effect of the therapy could be demonstrated whereas those of the VIENNA 8 strain reared in Seibersdorf become sexually mature at 2-days

of age and no effect of the therapy could be demonstrated. No field cage tests were conducted with treated males from the VIENNA 8 GSS.

4.3.2 Topical application of methoprene in A. fraterculus

A preliminary experiment was conducted to determine if methoprene could accelerate sexual maturity of *A. fraterculus*. Methoprene or acetone as control was applied to the thorax of 3-day old laboratory males of *A. fraterculus* that originated from a colony maintained in Tucuman, Argentina. Four days later mating tests were conducted in small cages using 7-day old males and mature females 14 days old. Results have shown that more males treated with methoprene mated on day 7 in a 30 min period than the control (**Figure 28**). These data need confirmation and additional experiments are needed to examine competitiveness of flies of different ages in field cages.

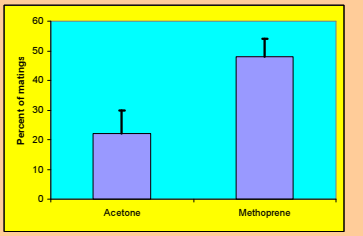


Figure 28. Effect of methoprene on mating in *A. fraterculus*

4.3.3 Effect of feeding methoprene to adult A. fraterculus

Methoprene was fed to newly emerged *A. fraterculus* males in the standard 3:1 sugar and protein adult diet. The controls for these experiments were males fed only sugar and protein. The size of the plural glands, which are the major sources of pheromone production in *Anastrepha* species, was selected as a parameter to measure the effect of methoprene on the acceleration and enhancement of sexual maturity and vigour. Results (**Figure 29**) showed that feeding methoprene to flies resulted in significant acceleration in development of pleural abdominal glands. Four day old treated males had pleural glands 75% the size of mature glands in control males and treated males 6 days old had similar size glands than mature control males 8 days old. Treated males 8 days old had glands 15% larger than in control mature males.

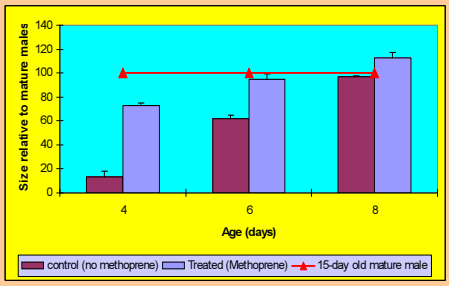


Figure 29. Effect of feeding methoprene on the size of the pleural abdominal glands in *A. fraterculus*

4.3.4 Composition of the male pheromone in A. fraterculus

To determine the chemical composition of male sexual pheromone of *A. fraterculus,* methoprene was fed to males in the 3:1 sugar and protein diet or the same diet without methoprene. The pheromone released by males was collected and analyzed chemically at the University of Florida. The volatiles released by males indicated the presence of the following compounds: bergamotene, suspensolide, farnesene, anastrephin and epianastrephin (**Figure 30**). These compounds are also pheromone components for other *Anastrepha* species. Although no reports on the pheromone are published we believe that these compounds constitute the major components because of their production by other *Anastrepha*

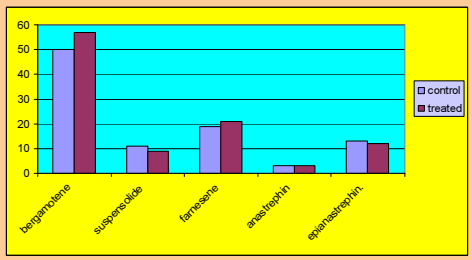


Figure 30. Volatiles present in the pheromone of *A. fraterculus*

species. Only one individual treated and control sample could be collected each day and only from 5 and 6-day old males. None-the-less, the data suggest that hormone therapy increased pheromone release (**Figure 31**). Additionally, the ratio of components in pheromone released by males was the same for treated and control fed males.

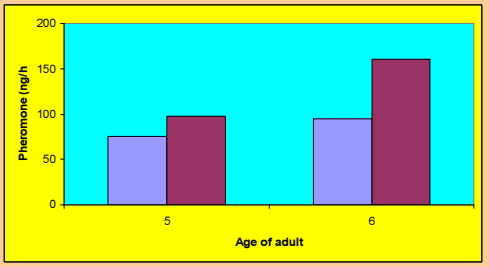


Figure 31. Pheromone release in treated (red) and untreated (purple) *A. fraterculus*

The results from the study on *A. fraterculus* are preliminary because only a few replicate samples were obtained. However, the data indicate that hormone therapy will accelerate reproductive development of *A. fraterculus* males. Further studies are required on mating behaviour to determine the age at which wild and sterile males become sexually mature; the effects of application of methoprene on acceleration of reproductive development; determination of the formulation and optimal dose required, and establishment of the application period and method required.

4.3.5 Methyl eugenol treatment of Bactrocera dorsalis

In Hawaii, Don McInnis and his group have succeeded in isolating potential genetic sexing strains (GSS) based on white pupal mutations for *B. dorsalis* and *B. cucurbitae*. In a collaborative project these strains have now been transferred to Seibersdorf for further studies on stability and competitiveness. In Thailand, an SIT programme is being implemented in areas where *B. dorsalis* and *B. correcta* are present and both species are being reared and released. There is obviously great interest in improving efficiency by introducing a GSS for *B. dorsalis*. In order to assess this possibility and reconfirm the effectiveness of methyl eugenol (ME) treatment on the enhancement of the sexual activity of treated sterile males, pupae from a field population of *B. dorsalis* were sent to Seibersdorf from Thailand and field cage compatibility studies have been carried out by D. McInnis using the GSS.

The GSS from Hawaii is ca 10 years old, but is derived from a 25 year old laboratory strain collected from papaya in Hawaii. These flies were tested when 10-20 days old, either non-irradiated or irradiated with 100 Gy under hypoxia. The “wildish” flies came from a laboratory adapted strain from Thailand that was 2 years old and had been through 17 generations of rearing (G17). Wild flies were collected in early 2005 in Thailand from natural host and shipped to Seibersdorf where they were reared for one generation on mangos as a natural host (G1). For the G17 strain 2-3 week old flies were used in the test and for G1 flies 4-5 week old flies were used. This ensures that all the strains tested were sexually mature.

Two field cages inside a greenhouse were used for the tests. Male flies (100-200) were exposed to 1ml of ME and allowed to feed on it *ad libitum* for 2 hrs in a ventilated 1 litre container. The ME was applied to a 5 cm strip of filter paper and hung from the top of the container. Male flies, either wildish or GSS, on alternating days, were painted by gently aspirating the flies into a net bag and then applying a small spot of white paint to the dorsal thorax of each fly. Test flies were held in the standard 1 litre containers with food (sugar and hydrolyzed yeast protein in a 3:1

ratio) and water until the test day. The ME application and any paint marking was done the day before the flies were used in a test.

At 17.00 on the day of the test, the male flies were released at the base of each of the two trees within each cage. Dead or non-flying flies were replaced with healthy flies, always maintaining the number of laboratory males equal to the number of wild males (usually 100). Approximately 30 min later, the females were released in a similar manner and replacements made, as before. Mating pairs were collected in clear plastic vials for several hours during the evening until none were observed for at least 30 min, at which time the test was concluded. Based on the numbers of laboratory male vs wild male matings, a Relative Sterility Index, RSI, was calculated for each cage. This index measures the competitiveness of laboratory males based on the proportion of wild females mated to laboratory males.

For the first experiment 5 replicates were conducted and non-irradiated and irradiated GSS males were compared against the G17 strain. For the second experiment, due to a limited number of flies, irradiated GSS males with or without ME were competed against wild G1 males and females.

At least 20%, and usually 40-60%, mating success was obtained from each cage over all of the test dates. In experiment 1 where G17 flies were used the relative RSI's varied considerably; however, the relative values for the treatments for any given day remained fairly consistent. RSI's averaged 0.28 for non-irradiated males, 0.20 for irradiated males without ME, and 0.41 for irradiated males with ME (**Figure 32**). Using the value for the irradiated GSS (without ME) as a standard (1.0 competitiveness), the treatment with ME had a doubling (2.05) effect on mating competitiveness. Irradiation alone reduced the mating success of GSS males ca 29%, on average, in the first 3 replications. In experiment 2 the mating performance of the GSS against the G1 strain was poor in the single test run (RSI=0.20). Only 7 matings out of 45 possible were recorded for the non-ME treated GSS, and all were by G1 males. Clearly, more replications with G1 material are needed.

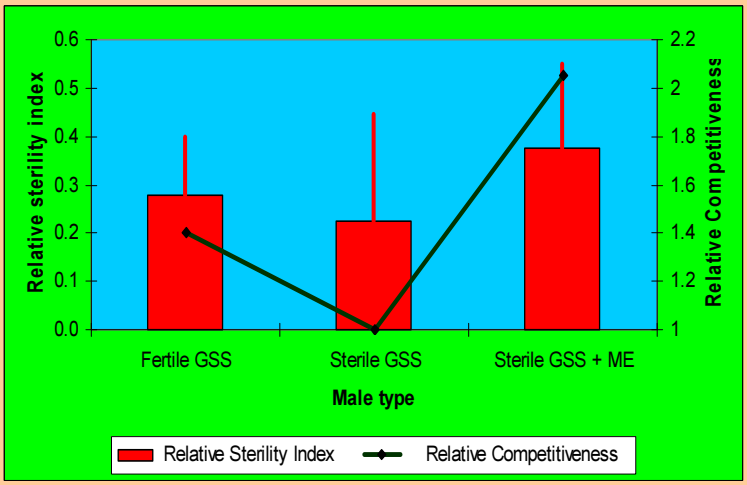


Figure 32. RSI and competitiveness of B. dorsalis GSS males

Figure 33. *B. dorsalis* from Thailand (left) and Hawaii (right)

During the course of the test period, the observers in the field cages noted that the body colour of the Thailand G17 and G1 flies was quite dark compared to the

body colour of GSS males (**Figure 33**). This darker colour was especially noticeable in the abdomen of the flies. This difference is in addition to the difference in thorax colour present in the GSS strain (white vs the normal yellow colour). This thorax colour trait is unrelated to the *wp* mutation since the white thorax colour is fixed in the standard non-sexing strain from which the sexing strain was derived years ago in Hawaii. This difference in body colour should be further investigated by comparing the Hawaii and Thailand populations genetically, especially their DNA profiles.

The following main points can be gleaned from these studies: (1) for the pupal colour-based GSS of *B. dorsalis* from Hawaii and reared successfully in Seibersdorf, an average reduction in male mating competitiveness of 18-36% due to irradiation by itself was observed. This result is based on tests of the GSS against the G17 strain, (2) a 2-fold beneficial effect of treating the GSS males with ME was demonstrated and (3) irradiated GSS males did not mate well with the G1 strain in the single test we completed. In this test, only ME treated males succeeded in mating with the G1 females. Clearly, this result needs to be followed up in further tests.

In conclusion these preliminary results have shown that GSS are compatible either with G1 or G17 flies from Thailand. When sterile males are fed with ME one or two days before being tested, their sexual performance was increased by a factor of 2. This result reconfirms the effectiveness of ME treatment on *B. dorsalis* sterile males and provides some confidence that the *B. dorsalis* white pupae GSS could be introduced and used for the SIT programme in Thailand.

4.4 Codling Moth *Cydia pomonella* Mating Compatibility

Controlling the codling moth is very important for the success of the pome fruit industry. In 1992 an area-wide SIT programme was established for first time in the Okanagan valley in British Columbia, Canada and continues to effectively suppress this pest in a large part of the valley. The pome fruit industry is very widespread and there is increasing interest in developing the SIT for the codling moth in many parts of the world, e.g. Brazil, Argentina and South Africa.

In comparison to fruit flies, codling moth rearing is quite complex and expensive and it may be possible in the future to supply different programmes with sterile moths from a few facilities. In codling moth this could be very effective as the species is present in temperate regions and undergoes diapause. A prerequisite for this concept is that codling moths from different parts of the world show mating compatibility. In collaboration with consultants from Argentina, Mr. Mario Sevilla and Mr. Gustavo Taret, field cage studies were carried on different geographical populations of *C. pomonella* in support of a CRP entitled “Improve Components of Codling Month SIT to Facilitate the Expansion of Field Application”. Excellent collaboration was received from colleagues who collected wild diapausing larvae for these experiments and also provided us with material from their colonies. The populations tested were:

1) SWI, Switerzand, (from a commercial company)
2) CC, Canada, (from the rearing facility in British Columbia)
3) CHI, Chile, (diapausing larvae from a wild population)
4) SYRW, Syria (diapausing larvae from a wild population)
5) NC, New Zealand (from a laboratory colony)

6) NW, New Zealand (diapausing larvae from a wild population)
7) ARW, Armenia (diapausing larvae from a wild population)
8) AW, Argentina (diapausing larvae from a wild population)

Figure 34. Codling moth larvae, males (left) and females (right).

The crosses that were assessed are shown in **Figure 35**. Experiments were conducted in field cages inside of green house. Preliminary laboratory experiment indicated that codling moth males and females reach sexual maturity 2 days after emergence but that they can mate within a few hours of emergence. This necessitated that 3rd instar larvae were sexed (**Figure 34**) to ensure that virgin moths were used in the field cage tests. 50 virgin couples of each population were released in the field cage one hour after the sun set and the number of couples was recorded every 30 minutes. The day before the test the moths were dyed using fluorescent powder in order to identify the different populations.

Results based on Isolation Index (ISI) (**Figure 35**) have shown some evidence mating isolation between some of the crosses e.g. CHI x CC, SYRW x NC and ARW x NW. All the other crosses showed no evidence of assortative mating. These quite extensive studies support the concept of shipping sterile moths from a central facility to release programmes in different parts of the world as is done for sterile fruit flies.

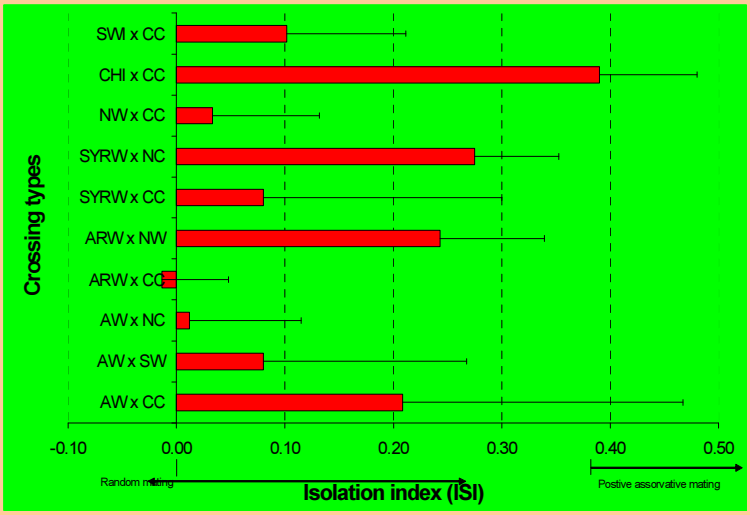


Figure 35. Isolation index between codling moth populations from different parts of the world

4.5 Optimal Larval Density for Colony Maintenance for VIENNA-8 GSS

During the three months of the fellow ship of Mr. Nathan Vermeulen and Mr. Hilton Asia from South Africa experiments were conducted to reassess the mass rearing protocol for the maintenance of the colony for VIENNA 8 GSS. All GSS for medfly are currently based on a reciprocal chromosomal translocation between the male determining chromosome and the autosome number 5 that carries the wild type genes for the selectable markers *white pupae* (*wp*) and *temperature sensitive lethal* (*tsl*). In these strains individuals generated following adjacent segregation carry

unbalanced chromosomes and generally show low viability. They frequently die during embryo, larval, pupal or adult stage depending on the proportion of the chromosome segments involved in the translocation responsible for the genetic sexing effect and this requires different mass rearing protocols for each specific strain. The objective of the evaluation was to assess the effect on pupal production and adult quality of different egg density rates as well as different amount of diet per larval tray.

The variables measured in this experiment were efficiency (pupal recovery from given number of eggs), the expected pupal production from one tower of trays (24 trays per tower) and the cost of pupae based on the cost of diet (0.50 US$ per kg of diet) and efficiency. The results from each treatment and for the variables evaluated are shown in **Table 9**. The main conclusions of this experiment were that density rate of 0.8 ml of eggs/kg of diet and trays loaded with 4 kg of larval diet (Treatment B3) produced acceptable efficiency for egg to pupae, high production of pupae per tower of trays and with the lowest cost per million of pupae (**Figure 36**).

Table 9. Different treatments result of the combination of 3 different eggs density in 3 different amount of diet per tray.

Treatment	Diet/tray (kg)	Eggs/kg of diet (ml)	Eggs/tray (ml)	Efficiency egg to pupae	Cost per million (US$)	Pupae per 1 tray tower (million)
A1	3	0.50	1.5	0.52	76.9	0.94
A2	3	0.64	1.9	0.40	79.0	0.91
A3	3	0.80	2.4	0.44	56.2	1.28
B1	4	0.50	2.0	0.45	88.4	1.09
B2	4	0.64	2.6	0.41	76.1	1.26
B3	4	0.80	3.2	0.44	56.6	1.70
C1	5	0.50	2.5	0.47	85.5	1.40
C2	5	0.64	3.2	0.38	81.3	1.48
C3	5	0.80	4.0	0.36	69.3	1.73
D1	6	0.50	3.0	0.44	91.4	1.58
D2	6	0.64	3.8	0.36	85.8	1.68
D3	6	0.80	4.8	0.34	73.2	1.97

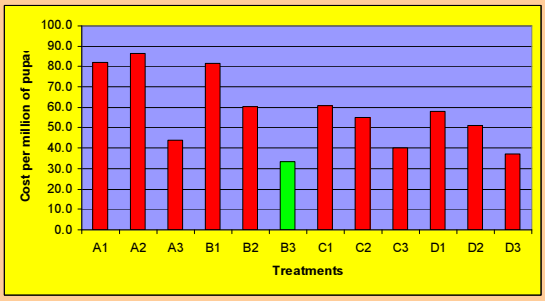


Figure 36. Cost of production of 1 million of pupae considers production efficiency and cost of larval diet ingredients.

5. Mosquito R and D

The provision of a suitable blood diet to large numbers of adult female mosquitoes will be major concern for any large scale mosquito SIT programme as it is for tsetse SIT. There are two components to this; firstly, the blood itself and secondly the manner by which it is presented to the mosquitoes. The use of live animals as a blood source has major logistical problems and from the beginning of the project this approach was not considered. This focuses attention on the use of membrane feeding and blood collection. Studies are described in this report which go some way to solve these issues but a reliable and effective way to achieve large-scale blood feeding still needs to be developed.

Excellent work has been carried out by the counterparts in Sudan in relation to the collection of base-line data from the project areas. Both entomological and epidemiological surveys have been carried out on a regular basis and a GIS-,based database has been established. Weather monitoring stations have been set up in the two field sites and many months of data have been successfully downloaded. A population genetic survey has been completed and it concluded that there is no substantial structuring in the target populations.

Marking mosquitoes before release using fluorescent powder, as is done for most SIT programmes for other species, has disadvantages related to the effects of the powder on the fitness of these fragile insects. A new method using stable isotopes was evaluated in 2005. By adding ^{13}C to the water in which the larvae were reared, the mosquitoes could be stably labeled throughout their adult life. This method, although simple to implement,t does have the disadvantage that identification of the label is expensive although new technology will certainly reduce the price in the future.

Germline transformation may provide one way to develop genetic sexing strains for An. arabiensis*, although problems with the release of such insects will remain. So alongside studies using the classical approach, transformation technology is being developed. Results at the end of 2005 were very encouraging using DNA constructs which should selectively label the sperm in adult males. Although this type of label may not be useful for genetic sexing* per se*, it could be very useful for determining the mating status of female mosquitoes.*

5.1 Use of ^{13}C as a Population and Resource Marker

In SIT programmes it is important to follow the progress of sterile to wild insect ratios in the field. This is usually done using mark recapture techniques, although there are many marking techniques available, few are suitable for mass rearing and mass release. There are a number of criteria for effective marking of insects: a) retention, b) not affecting the insect's behaviour, c) durable, d) non-toxic, e) easily applied, f) clearly identifiable and g) inexpensive. Among the methods available for mass marking of insects are dye marking, genetic marking and chemical marking. One of the problems associated with dye marking is that it is often less than 100% effective and in addition the action of application of the dye can reduce fitness of the adult population especially in small fragile insects such as mosquitoes. Introduction of a genetic marker into a sterile population may cause ethical concerns and detection at a molecular level is time consuming and costly. Labelling of populations with stable isotopes was proposed as a possible method of marking of mosquitoes. Stable isotopes are naturally abundant in the environment, safe and non-radioactive. Stable isotopes have the same atomic number, but a different number of nuclei and thus a different atomic weight; therefore they react chemically identically to the more common isotope. A series of experiments were designed to test the feasibility of using ^{13}C as an as a potential chemical marker.

There is also considerable interest in the role of sugar feeding in mosquitoes in terms of survival and reproductive success. As the sole source of food for males, it is of particular significance in terms of their ability to engage in mating activities. This behaviour is vitally important for strategies in which males are released in the field with the aim to effectively compete with wild males for virgin females. Moreover, sugar feeding is routinely used for mosquito colony maintenance. Nutrient allocation has been difficult to measure quantitatively in most insects, but stable isotopes have proved to be a useful tool in studying the uptake and turnover of nectar in hawk-moths. Using ^{13}C much additional information on the turnover of sugar feeding in male *An. arabiensis* was obtained.

Labelling was done at larval stages as it was felt that this would result in most uniform labelling and it would be feasible for both larval and adult release strategies. Labeled-^{13}C glucose was incorporated into the larval diet in a powder or liquid form. ^{13}C enrichment in the emerged adult mosquitoes was monitored over the adult life span of the mosquito. The isotopic enrichment of the adult male mosquitoes declined as it switched from a labelled larval diet to an unlabelled adult diet due the natural turnover of carbon through

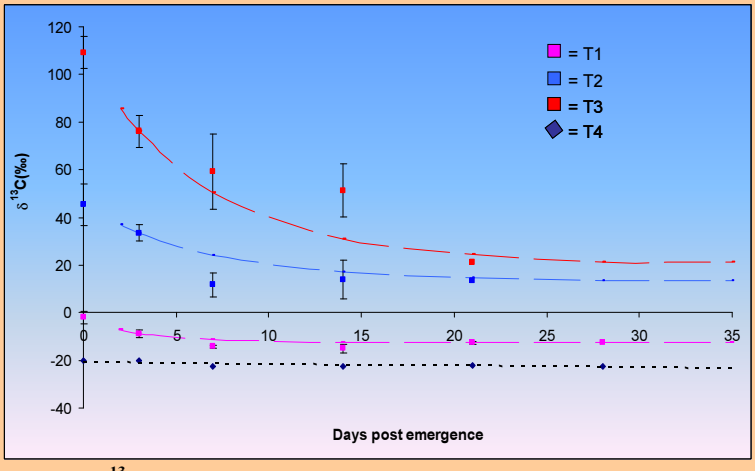


Figure 37. ^{13}C of whole mosquito against time after emergence (days) in for treatment T1-T4. Bars are +/- 1 standard deviation. The dashed lines are the modelled data. T1-T3, 0.5, 2.5 or 5 mg of 99 atom % ^{13}C dried ground glucose per gram of fish food, mixed dry, T4 control unlabelled fish food only.

respiration and feeding. This decline in enrichment allowed calculation of the contribution of adult sugar feeding to the total mosquito carbon pool and determination of the size of the metabolically active carbon pool. The decline in the adult isotopic enrichment was monitored by destructive sampling of whole mosquitoes and analyzed using an elemental analyser linked to an isotope ratio mass spectrometry. Sufficient label was incorporated into the adult structural tissues and was retained at a detectable level throughout the mosquito's adult life (**Figures 37 and 38**). Metabolic turnover of the adult male mosquitoes was described using a two-pool model of mosquito isotopic composition (**Figure 39**) which consisted of dynamic carbon pool and a static carbon pool. The proportion of the total adult carbon pool derived from the adult sugar diet over the life span of mosquitoes was determined and the ratio of structural carbon, with a low turnover rate, to metabolically active non-structural carbon, was assessed. The uptake and turnover of sugar in the metabolically active fraction suggests that after 3 days >70% of the active fraction carbon is derived from sugar feeding (increasing to >90% by day 7), indicating the high resource demand of male mosquitoes.

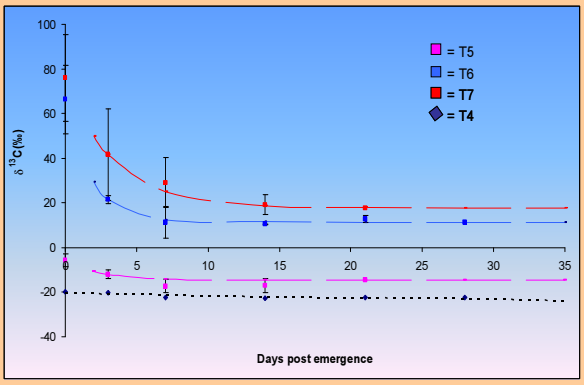


Figure 38. ^{13}C of whole mosquito against time after emergence (days) in for treatment 4-7. Bars are +/- 1 standard deviation. The dashed lines are the modelled data. T5-T7, 0.175, 0.875 or 1.75 mg of 99 atom % ^{13}C glucose added to trays as a one shot solution in addition to regular

It was possible to mark adult male mosquitoes by the addition of labelled glucose to the larval diet. The isotope label was sufficiently "fixed" in the adult, i.e. enough label was incorporated into the structural tissues, and was retained at a detectable level throughout the mosquito's adult life. The average enrichment minus the standard deviation of the adult mosquitoes was high enough in treatments T2, T3 T6 and T7 and at all sampling times greater than 0.0 ^{13}Ç‰ vs PDB a value which is rarely observed in terrestrial organic resources in nature and would thus confirm it as an artificially-labelled specimen. After 3 days, 31 % of the adult mosquito carbon was derived from the sugar water fed to the adult mosquitoes, whilst after 7-10 days this figure levelled out to around 50%. It was

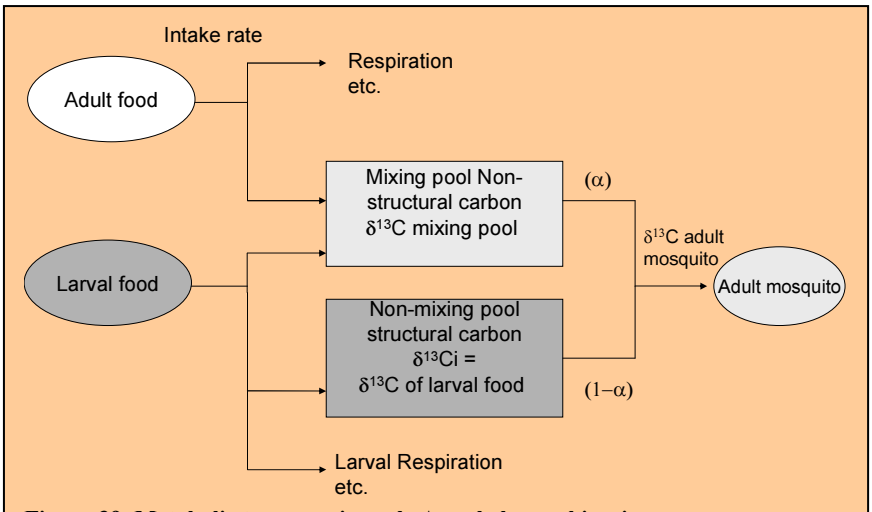


Figure 39. Metabolic turnover in male Anopheles arabiensis

possible to "fix" the isotopic label in adult *An. arabiensis* and to detect the label at an appropriate concentration up to 21 days post-emergence. Given the scale of SIT or mark-recapture studies and the operations and the logistics involved, the cost of the label for marking purposes would not be prohibitively expensive. Based on the data from treatment T6 cost of labelling would be less than 250 US$ per million mosquitoes labelled. Costs of isotope analysis can be as low as 5 US$ per sample and may come down further given the current developments in laser technology. In addition, sample preparation for analysis is minimal as the whole mosquito is put into the tin cup and analysed, these samples are then dried and once the tin cups are sealed can easily be transported for analysis.

5.2 Investigation of Improved Adult Sugar Diet

Experiments were conducted to determine the effect of a sugar meal preservative on adult mosquito longevity as determined by Kaplan-Meier survival analysis. The standard 10% sucrose meal was compared with one also containing 0.2% methyl-4-hydroxybenzoate (methylparaben). Meals were changed weekly and each cage contained 30 females and 30 males. Significant increases in the longevity of both sexes of *An. arabiensis* were observed.

We asked whether the beneficial effect on longevity was due to a direct effect on the mosquitoes or an indirect beneficial effect on the mosquitoes by affects on the sugar meal. An experiment was conducted in which sugar stored at 4ºC was changed daily or 10% sucrose containing methylparaben stored at room temp was changed once a week. The indirect effect is implicated: survival in the two groups was indistinguishable. It was concluded that some method – either frequent meal changes or use of a preservative – will be beneficial for adult broodstock longevity and should be implemented.

Additional experiments using the stable isotope ^{13}C as a label in choice tests with *An. arabiensis* demonstrated that the meal containing sucrose alone is preferred by both sexes over the meal containing methylparaben. Therefore, the less-preferred meal is the one that most increased adult longevity, an observation that demonstrates that adult preference is not always the best indicator of conditions that are suitable for insectary culture.

5.3 Inherited Sterility

In some insect species, radiation with a less than optimal sterilizing dose produces F1 offspring that can show quite high levels of sterility. Although in Diptera this effect is not so significant, it may be important in mosquitoes if fully sterilizing doses cause too high levels of somatic damage. The offspring of the adult irradiation experiments were used to study the effect of radiation on inherited sterility of the F1 generation. F1 males and females were separated to sex and mated with normal mates. A blood meal was offered by membrane or hand, eggs were collected *en masse*

Table 9. Inherited sterility of the F1 generation (male and female) of irradiated males

Sex (F1 generation)	Treatment	# Eggs	Hatch (± SE)
male	0	3898	84.7± 0.4
male	25	2928	78.2± 0.9
male	50	3028	80.2± 6.0
male	60	1655	69.5
male	80	1056	64.2
male	100	458	49.6
female	0	6218	72.0± 1.2
female	25	3452	79.4± 1.0
female	50	4449	65.6± 9.0
female	60	1846	47.4
female	80	1593	65.3

and hatch rates were determined. Data on the level of inherited sterility is presented in **Table 9**. There is a trend to a reduced hatching rate at higher doses, especially in males, but more data is required.

5.4 Competition Experiments

Two pilot competition experiments were initiated to gain insight into the competitiveness of irradiated males. 30cm^2 laboratory cages were used for the experiments. Males, <24hrs or >24hrs of age, were irradiated with 70 Gy, and introduced into a cage with normal males and normal females at different ratios. Females were fed on a volunteer, egg masses collected and en masse ovipositioning in the cage occurred, and hatch rates were determined. **Table 10** shows the observed hatch and level of competitiveness present. On a 1:1 ratio, competitiveness is decent, with values between 0.56 and 1. Between equal treatments of one experiment (Exp 2, 1:1 ratio) there is some variation observed. At the higher ratios of sterile males there is large variation in level of competitiveness observed. This data stresses the need for further studies, which will be initiated in 2006 and will be performed in larger cages.

Table 10. Level of competitiveness of KGB males irradiated with 70 Gy. Ratio is expressed as irradiated: normal males. Expected hatch and competitiveness are calculated according to Fried's index.

Experiment	Age ♂ irradiated	Ratio: I:N	# Eggs	Hatch		Competitiveness
				Observed	Expected	
1	< 24	1:0	554	77.44	-	-
1	< 24	1:1	782	50.06	41.22	0.61
1	< 24	3:1	767	43.42	23.11	0.30
2	< 24	1:1	965	49.83	41.50	0.63
2	< 24	2:1	909	9.36	29.33	7.87
2	< 24	5:1	858	31.79	17.17	0.35
2	> 24	1:1	829	51.81	41.50	0.56
2	> 24	1:1	1238	41.32	41.50	1.01

5.5 Individual Egg Laying

Experiments were undertaken to increase the number of females ovipositing individually. In KGB, routinely around 75% of the females deposited eggs after a blood meal; in Dongola this number was lower and around 50%. A positive correlation was found between batch size and hatch rate in KGB ($r = 0.304$; $p < 0.01$), but the correlation coefficient explained < 10% of the total variation.

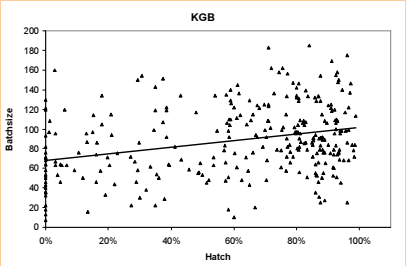


Figure 40. Hatch versus batch size in the KGB strain

The graphs clearly demonstrate the large variation in hatch rate observed in the batches of KGB females (**Figure 40**). This variation is much lower in Dongola (**Figure 41**), resulting in higher average hatch rates compared to KGB ($t(400) = -6.6$, $p < 0.01$). Variation in hatching rates between experiments was observed in KGB. In experiment 1 lower hatch rates were observed than in the 2nd and 3rd experiment ($F_{2,294} = 27.4$, $p = < 0.01$, data not shown).

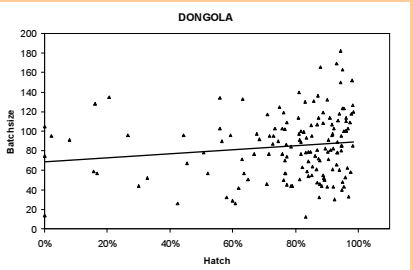


Figure 41. Hatch versus batch size in the Dongola strain

5.6 Blood Feeding

Colonies of *An. arabiensis* are maintained by feeding adult female mosquitoes with human blood twice weekly. The relatively small size of the colony kept exclusively for research purposes means that the dietary requirements are proportionally modest; 36ml of blood is drawn weekly from laboratory staff to sustain the colony in Seibersdorf.

For mass production like the magnitude required for SIT, where millions of mosquitoes are produced weekly, a much greater volume of blood is required. Live animals are used widely as blood source for routine laboratory maintenance. While this comes with the advantage of feeding blood in a natural condition, the drawbacks of keeping live animals in the laboratory could not be discounted. The nursing of live animals will incur additional demands for space, time and manpower. Also, severe stress is placed on the animals by the feeding, thereby increasing the potential for induced illness due to the feeding stress or due to subsequent crowding in the holding cages. There is also the necessity for shearing and preparing the animal for feeding and special equipment is needed for holding the animal during the confinement in the mosquito cages. In mass rearing for SIT, the sheer size of the colony will have 2 concomitant demands on the blood feeding procedure: (1) a substitute to human donors; and, (2) a satisfactory storage method that retards the physical and chemical processes that render stored blood inferior to fresh blood. Furthermore, to ascertain the quality and the number of mosquitoes produced, blood feeding, as all aspects of rearing, need to be standardised.

Blood feeding experiments are being carried out in two phases: (1) the initial phase identified the effects of storage on the female feeding response that was determined by scoring the number of individuals that probed against the numbers that fed and engorged. Oviposition rates of females that were fed with 5, 4, 3, 2, 1 day-old and fresh blood (>6 hour from the time of withdrawal) were compared. Results from these experiments confirm the preference of mosquitoes to fresh blood; there was an observed decline in the feeding response and fecundity in females fed with older blood. In a separate experiment, the effects of mixing old and new blood was investigated. Results show that more eggs were oviposited by mosquitoes fed with a mixture of 4-day old and fresh blood (>6 hours old) than by females fed with 4-day old blood alone. Similarly, mosquitoes fed with 5-day old blood and fresh blood laid more eggs than those fed only with the 5-day old blood. Human blood used in these experiments were collected and stored in heparin-coated Vacuettes®.

5.6.1 Membrane feeding

The Hemotek® 5W1 membrane feeding system was used in the feeding experiments (Discovery Workshops, Lancashire, UK). The system includes 5 FU1 feeders with a built-in temperature control and heater. Each feeder has a detachable meal reservoir that holds up to 5ml of blood. A piece of collagen membrane is stretched over the aperture of the meal reservoir and secured with a rubber ring. Blood is poured into the reservoir through 1 of the 2 openings. Once the reservoir is filled and air bubbles are expelled, the holes are sealed with plastic plugs to prevent the contents from

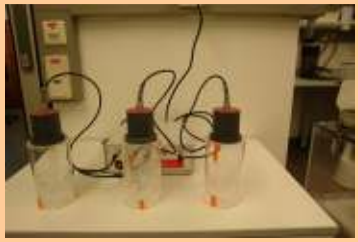


Figure 42. Haemotek blood feeding system

spilling out of the receptacles. The reservoir is then screwed to the heating plates. Blood is warmed to 37.5°C before the feeders are placed on the tubes holding the experimental mosquitoes.

5.6.2 Blood storage

The next step would be to investigate the response of females to bovine blood preserved in different anticoagulants: sodium citrate, acid sodium citrate, citrate phosphate dextrose adenine (CPDA-1), ethylenediaminetetraacetic acid (EDTA), and heparin. The aim of these experiments is fourfold. First, to test the suitability of bovine blood as a valid substitute to human blood, which is currently in use in the Seibersdorf insectary. Secondly, to ascertain the choice of anticoagulant for storage; Thirdly, to establish the most efficient storage protocol, and; Fourthly, the experiments will determine the allowable storage time for bovine blood. Artificial blood feeding and storage are expected to have a combined effect of a reduced response and lower fecundity in laboratory-grown *An. arabiensis.* An efficient feeding regimen would abate the negative results of artificial membrane feeding and storage. In addition, the feeding response and the fecundity rates of colonies fed in this way should be comparable to colonies fed by natural means. Through our experimentations we hope to gain a posteriori knowledge on how best to artificially feed mosquito colonies with stored bovine blood. Insight gained from these experiments will be valuable contributions to our existing knowledge of mass rearing *An. arabiensis* for SIT.

5.7 Mosquito Handling and Housing

In the ANNUAL REPORT 2004, a concept design for a cage to house large numbers of mosquitoes was described; the cage has now been built and is undergoing evaluation. The goal was to have cage that could hold enough adults to produce the number of eggs required for 100,000 sterile males a day, but it also had to be designed so that the four essential activities: blood and sugar feeding, introduction of pupae and removal of eggs could be carried out without opening the cage. Ideally the cage should be capable of operation with a fixed number of adults without disassembly for cleaning. The entire cage has a footprint of 1 x 1 m, and a height of approx. 0.8 m. It is designed so that an identical unit or units can be joined end to end for modular expansion (**Figure 43**). The cage also incorporates features that are similar to the mating cage designed by Ron Marchand that includes an artificial horizon and unbroken artificial sky to promote anopheline mating.

Figure 43. Adult mosquito holding cage

The cage has sloped sides and louvers for daytime resting to reduce defecation of adults on one another. The floor is disposable paper which can be replaced by feeding it through a slit. The four essential activities described above are performed through 60 mm diameter tubes that insert from outside of the cage (**Figure 44**). Pupae can be poured into a tube, and the emerging adults escape from a slit 6 mm wide. We have demonstrated that this size does not prevent adult escape but neither does it provide an attractive – and

Figure 44. Access tubes for mosquito holding cage

undesirable - opening for female oviposition. Sugar water is provided through a rectangular cross-section plastic tube which has openings on the bottom covered with 20 micron opening polyester mesh. This size has been proven capable of repeated use, does not allow dripping and adults can feed through it easily.

Figure 45. Transferring and counting adult mosquitoes

The survival of adult mosquitoes following their transfer from emerging trays to rearing cages was significantly improved with the development of a vacuum-based cage loading system. This device is the result of a fruitful collaboration with the Instrumentation Unit (Mr. Gardos) and the mechanical workshop (Mr. Nirschl and his team of engineers) of the IAEA laboratories. A rearing cage is placed in a tight plexiglass box maintained under negative pressure (**Figure 45**). Newly emerged adult mosquitoes are gently suctioned from the larval tray into a standard adult rearing cage. This system was equipped with an optical counter to keep track of the number of adult mosquitoes loaded into the cage. Used for the handling of delicate adult mosquitoes and the accurate assessment of daily production in the laboratory, this system and its simple design will be easily scaled up and automated to accommodate much larger mosquito production regimes.

5.8 Germline Transformation as a Supporting Technology for Genetic Sexing

Parallel to the more conventional approach to genetic sexing (based on radiation-induced translocation of a resistance marker to the male-determining chromosome) efforts are underway to achieve genetic sexing of *An. arabiensis* using a transgenic strategy. While *An. gambiae*, the major vector of human malaria, has previously been transformed using the *piggyBac* vector, significant difficulties mostly related to handling of injected specimens have not only hindered successful application to other members of the *An. gambiae* complex but also the rapid spread of such technology across research institutions. Confronted with such challenge, we have increased our interactions and exchange of information with other laboratories in Europe and the USA that are also involved in the development of transgenic mosquitoes. As a result new collaborations have been established, particularly with the laboratory of Dr. Crisanti at Imperial College, UK. Such collaboration has lead to the recent testing of a *piggyBac*-derived construct specifically designed to facilitate the development of a transgenic sexing strain. Following Ms Labbé's completion of an MSc degree and her subsequent departure from the mosquito project, Mr. Thailayil was recruited to assist in this effort. Injections using the *piggyBac* are now being performed on the *An. arabiensis* Dongola strain which was successfully colonised and transferred to Seibersdorf in 2004. While testis-specific GFP expression is readily observed in *An. arabiensis* mosquito individuals injected with the *piggyBac* vector (**Figure 46**), an indication that the plasmid DNA was properly delivered during the injection, transgenic lines are yet to be obtained from back-crosses with these fluorescent specimens. Future efforts toward establishing a genetic sexing strain will be focused in verifying the activity of the *piggybac* helper plasmid in *A. arabiensis* using an *in vivo* mobilization assay.

Figure 46. *A. arabiensis* larvae displaying somatic, testis-specific GFP expression.

5.9 Larval Phenotypes

An. arabiensis mosquitoes display various phenotypes that can be distinguished at the larval stage. Keeping track of their frequency in colonies can be a useful tool to evaluate their genetic make-up. Larvae of the KGB, Dongola and Sennar strains were carefully screened during the various stages of their development. In addition to three well-described phenotypes (*collarless c+, collarless c/c*, and *red stripe*), two other known phenotypes (*black diamond*, *green body*) and one potentially new phenotype (*blond punk*) were documented (**Figure 47**). A screen of the three colonies revealed that there were discrete differences between them regarding the presence or absence of these larval phenotypes.

Figure 47. *collarless c+* and *c/c* KGB larvae.

5.10 Development of a mtDNA PCR Diagnosis for Strain Identification.

The ability to distinguish between various strains of *An. arabiensis* maintained in the Unit represents an essential component of quality control. A molecular-based discrimination tool was developed using restriction analysis of PCR-amplified regions of the mosquito mitochondrial DNA (mtDNA). Following the testing of PCR primers already described in various phylogenetic studies, three sets of primers were selected to amplify large areas (4-6kbp) of the *An. arabiensis* mtDNA (15kbp). These PCR products were then characterised using five restrictions enzymes and screened for loss or gain of restriction sites corresponding to strain-specific mtDNA rearrangements. This analysis of long, PCR-amplified mtDNA fragments lead to the successful identification of differing mtDNA restriction patterns between the Dongola and KGB strains following *Eco*R1-*Hind*III digestion of a 3.9 kb PCR fragment (**Figure 48**). These two strains can now be easily distinguished at molecular level with minimal effort.

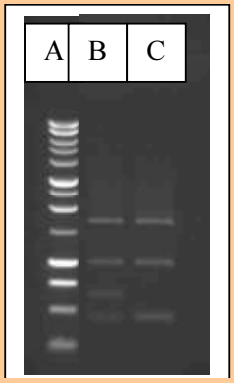


Figure 48. Restriction patterns between the *A. arabiensis* KGB (B) and Dongola (C) strains. DNA marker (A).

5.11 Field Site Activities

5.11.1 La Reunion

Following a meeting of the scientific steering committee in Vienna in December 2004, a document was drafted to define the preparatory research programme for the elimination of *An. arabiensis* from the island of La Reunion using SIT. This document has been distributed via the French mission and the French Atomic Energy Commission (CEA) to the national and local institutions as well as international experts identified as potential partners in the proposed feasibility study. This initiative has generated renewed interest for the La Reunion project and a meeting sponsored by CEA was organized in November to which the main research institutions (Institut Pasteur, IRD and CIRAD) participated. The severe Chikungunya epidemics which increasingly affected people in La Reunion during 2005 also prompted our group to adapt the strategic plan by including *Aedes albopictus*, principal vector of the virus on the island, in the proposed mosquito SIT feasibility study. A document has recently been forwarded to the French authorities for consideration.

5.11.2 Sudan

During a mission in early 2005 extensive activities were carried out in the field site in the Northern State. These included 1) selection and evaluation of a number of candidate sampling grid cells to act as 'static' site for future field surveys in Merowe and Dongola, 2) identification of a suitable basis for stratifying the selection of random sites for future field surveys in Merowe and Dongola, 3) verification of the suitability of the current protocol for field data collection for both field sites, 4) evaluation of the training of key fieldworkers in the use of handled field computers and 5) collection of remote sensing ground truth data for the Merowe field area. The two static field sites selected for data collection and **Figure 49** shows some satellite images for the static block in Dongola. These images show potential larval breeding sites for *An. arabiensis*. The development of this GIS based base-line data collection system will greatly facilitate the future release of sterile mosquitoes in the field sites. Studies are also being carried out to determine the population structure of the mosquitoes in the field site using DNA analysis. Dr Colin Malcolm (Queen Mary, University of London) visited the study area at the end of March to help in selection of certain points located at specific distances for mosquito collection as part of the study of population genetics (**Figure 50**). DNA analysis of the populations in the target areas have revealed no significant levels of population structuring.

Figure 49. Potential An. arabiensis breeding sites in the static block in Dongola.

Figure 50. Collection sites for *An. arabiensis* along the Nile

Larval surveys started in March and were carried out every month for collection of more than 40 variables from static and random blocks using GPS/PDA system on Trimble computers. The rate of coverage was 15–20 blocks/day and the static blocks were changed every month. Dissolved oxygen, water temperature and salinity were measured using probes. Metrological data including minimum and maximum temperature, relative humidity, wind direction and speed were downloaded every four months from the four automatic weather stations installed in the two sites.

These surveys enabled a picture to be built up of possible larval breeding sites in both Dongola and Merowe and **Figure 51** shows these sites in Merowe.

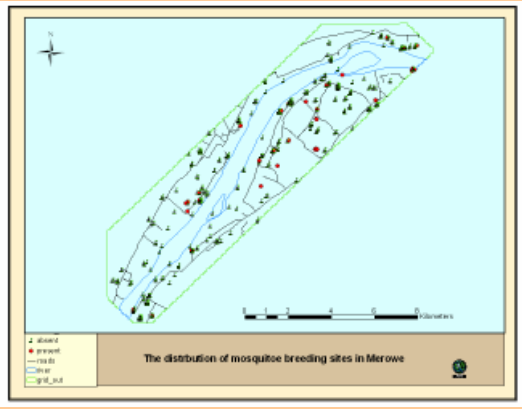


Figure 51. *An. arabiensis* breeding sites in the Merowe field site.

A large greenhouse was constructed in Dongola in November (**Figure 52**). The greenhouse will be used to study the biology and behaviour of the mosquitoes and to assess the competitiveness of sterile males. This is a very valuable addition to the field site and will enable field studies to be carried out using mosquitoes from the target population.

Figure 52. Large field cage constructed in the Dongola field site.

6. Appendices

6.1 Publications

ATKINSON, P.W., D.A. O'BROCHTA and **A.S. ROBINSON.** (2005)**.** Insect transformation for use in control. In Insect Pharmacology and Control – a volume in the series "Comprehensive Insect Biochemistry" Eds S.S. Gill, L.I. Gilbert and K. Iatrou. Vol 5, pp 403-411.

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MUTIKA, G.N. and **A.G. PARKER**. Induced sterility of *Glossina pallidipes* Austen males after irradiation in a nitrogen atmosphere. Entomological Science. (In press)

TOURÉ, Y.T., and **B.G.J. KNOLS.** Genetically-modified mosquitoes for malaria control: Requirements to be considered before field releases. In: "Genetically modified mosquitoes for malaria control" Ed C. Boëte. Landes Bioscience. (In press).

VAN DEN BOSSCHE, P., K. AKODA, B. DJAGMAH, T. MARCOTTY, R. DE DEKEN, C. KUBI, **A. PARKER** and J. VAN DEN ABBEELE. The effect of a single treatment of tsetse flies with isometamidium chloride on the fly's subsequent susceptibility to trypanosome infections. Journal of Medical Entomology (In press)

6.2 Travel

Staff Member	Destination	Period	Purpose of Travel
Bossin, H.	Geneva, CH	25-26 Jan	WHO meeting on GLP
	Crete, GR	24-30 July	Biology of Disease Vectors course
	French Polynesia	7-22 Nov	Prepare Gates proposal for *Aedes aegypti* control
Caceres, C.	Valencia, ESP	8-10 March	Advise on medfly rearing facility
	Tel Aviv, ISR	14-16 March	Review/advise/prepare work plan re medfly work (ISR/5/010)
	Quezon, PHI	28 March-1 April	Fruit fly mass rearing RCM
	Lima, PER	20-22 June	Advise on medfly rearing facility
	Florence, IT	26-28 Oct	IOBC meeting on olive fly
Franz, G.	Tapachula, MEX	22-23 August	Review project MEX/5/027
Helinski, M.	Moscow, Idaho, USA	10-15 April	Visit to Mark Klowden's lab to learn mosquito dissection techniques
Knols, B.G.J.	Paris, FR	12 Jan	Invited seminar at the Pasteur Institiute
	Khartoum, Dongola and Merowe, SUD	10-22 Feb	Discuss current activities and field missions to Dongola and Merowe .
	Amsterdam, NL	28 April	Fungus against malaria meeting
	Stockholm, SWE	17-18 May	Invited seminar
	Oxford, UK	27-29 July	Visit to Oxitec and invited seminar
	Reno, Atlanta, USA	3-11 Oct	SOVE conference
	Yaounde, CAM	10-18 Nov	MIM conference
Parker, A.	Bratislava, SLR	12 Jan	Technical assistance to IZ-SAS.
	Addis Ababa, ETH	24-28 Jan	Review facility construction
	Bratislava, SLR	16 Mar	Technical assistance to IZ-SAS.
	Bratislava, SLR	13 April	Technical assistance to IZ-SAS.
	Addis Ababa, ETH	23-25 May	Review facility construction
	Bratislava, SLR	26 May	Technical assistance to IZ-SAS.
	Pretoria, SAF	13-17 June	Advise on tsetse rearing facility
	Addis Ababa, ETH	19-23 Sept	Review facility construction
	Addis Ababa, ETH	26-30 Sept	Attend ISCTRC meeting
	Bratislava, SLR	25 Oct	Technical assistance to IZ-SAS.
	Port Lois, MAU	24-27 Oct	Advise on radiation protocols in relation to TC project MAR/5/015
	Bratislava, SLR	9 Nov	Technical assistance to IZ-SAS.
	Bratislava, SLR	7 Dec	Technical assistance to IZ-SAS.
Robinson, A.S.	Bratislava, SLR	12 Jan	Technical assistance to IZ-SAS.
	Addis Ababa, ETH	5-7 Feb	LTTRN Workshop
	Riverside, USA	7-11 March	Exotic Fruit fly symposium
	Rome, IT	4-8 April	ICPM meeting
	Montevideo, URG	30 May-3 June	Screwworm RCM
	Bratislava, SLR	14 Sept	Technical assistance to IZ-SAS.
	Bratislava, SLR	7 Dec	Technical assistance to IZ-SAS.
	Tapachula, MEX	14-16 Dec	Review project MEX/5/027

6.3 Fellows

Name	Country	# Months	Dates	
Ms. S.S. B. ALI	Sudan	6	2005-02-14	2005-08-13
Mr. O.M.E.M. SEID AHMED,	Sudan	6	2005-04-01	2005-09-30
Mr. N. VERMUELEN	South Africa	3	2005-08-01	2005-10-31
Mr. H. ASIA	South Africa	3	2005-08-01	2005-10-31
Ms. S. RADONJIC	Serbia-Montenegro	3	2005-10-01	2006-03-31
Ms. E.M.S. EL-KHOLY	Egypt	3	2005-10-03	2006-04-02
Ms. K.J. KIGODA	Tanzania	3	2005-10-06	2006-04-05
Mr. S.K. KEMBOI	Kenya	2	2005-11-01	2006-04-30

6.4 Staff List

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