ANNUAL REPORT 2003

ENTOMOLOGY UNIT
Agency's Laboratories
FAO/IAEA Agriculture and Biotechnology Laboratory
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1. Introduction

*As described in the **2002 Annual Report** an improved medfly genetic sexing strain (GSS) was developed, VIENNA 8. This strain shows improved production characteristics and will have a major impact on the production efficiency in SIT rearing facilities. During 2003 this strain was transferred to four rearing facilities in Guatemala, Peru, South Africa and Tunisia based on acceptable QC data developed for the strain in the Unit. For the first time the strain was transferred to the different facilities under an agreement that prevents further distribution to third parties without consent of the Unit. In the past, strains originating from the Unit have been freely exchanged within the medfly SIT community, sometimes with not always the best results.*

During 2003 there has been a substantial increase in the resources devoted to activities in fruit flies other than medfly e.g. Bactrocera oleae and Anastrepha fraterculus. Rearing of the olive fly, B. oleae has traditionally not been straightforward due to the very specific, and expensive, larval diet that is required by this monophagous pest. With the disappearance of an essential larval diet component from the commercial market an urgent search is underway for an alternative. In 2004, experiments will be carried out to identify improved larval diets. For A. fraterculus, a large-scale evaluation of mating compatibility between different geographical races has been carried out by a consultant.

Progress has been made in the containerised rearing of tsetse fly colonies. The container required considerable work before the temperature and humidity controls were suitable for tsetse rearing and a closed colony of Glossina fuscipes is now being reared. Discussions are now underway to install a version of TPU 3.2 (an automated tsetse feeding and production unit) in the container. A protocol for the handling, irradiation and eventual release of large numbers of sterile male tsetse has been formulated and it will involve at least two chilling periods, one during radiation and one during transport and release. A temperature regime to accommodate these procedures was developed and successfully tested. A preliminary experiment using infrared scanning to sex tsetse pupae was successful and further tests will be carried out in 2004.

In June 2003 the mosquito rearing laboratory was completed and a colony of Anopheles arabiensis established. Bart Knols joined the Unit as a staff member and he will be responsible for developing production and quality control protocols for mass rearing. Mark Benedict returned to CDC, Atlanta in October and is expected to rejoin the group in 2004.

Herve Bossin, Raimundo Braga and Teresa Vera spent several months in the Unit as consultants. At the end of 2003 two technicians, Harold Matzenauer and Gertie Germershausen had to leave the tsetse group as extra-budgetary funding was terminated.

1.1 Introducing *Wolbachia* into VIENNA 8

Wolbachia is an endosymbiotic bacterium present in the reproductive organs of many insect species. In some species, crosses between different populations are sterile due to the action of this bacterium. The bacterium has recently been introduced into the medfly and has been shown to demonstrate complete sterility when certain strains are crossed. A prerequisite to any use of this type of sterility in the field is that no female insects have to be released together with the males. Kostas Bourtzis (Univ. Ionina), who did the original introduction into medfly, visited the Unit and injected *Wolbachia* into the VIENNA 8 GSS. Strains derived from the injected embryos are undergoing analysis.

1.2 Transformation of Medfly

As part of the ongoing activities related to support of a new CRP on genetic transformation, Al Handler (USDA/ARS, Gainesville), has carried out further transformation experiments in the Unit. These experiments utilized new vectors that should lead to increased stability of the introduced transgene. A large number of transformed lines have been produced and are now being characterised. To further support these activities USDA has awarded a grant to Al Handler and part of the funds will be transferred to the Agency in 2004. Rossy Krasteva, who has been a consultant in the Unit for the past three years, is now working with Al Handler in Gainesville.

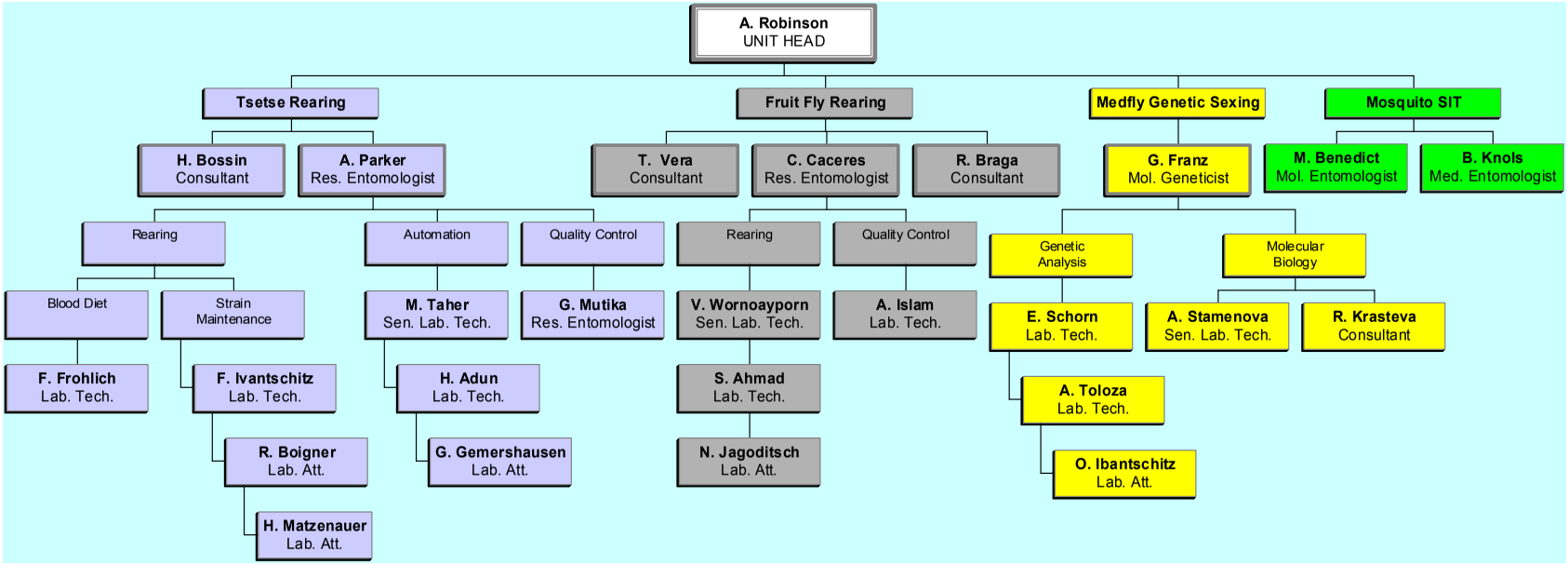
1.3 Olive Fly Rearing

The monophagous nature of the olive fly, *Bactrocera oleae*, presents difficulties when artificial diets have to be designed, this is especially so for the larval diet. In the 70's and 80's tremendous progress was made in artificial diets for olive fly but production costs were very high. In line with the current activities on olive fly in the Unit, new larval diets are being tested and alternative sources of expensive and increasingly unavailable products are being evaluated. Chronis Reboulakis (Univ. Crete) visited to Unit for 10 days to help with these activities. New diet preparation protocols were introduced as well as new oviposition substrates. The efficiency of olive fly rearing in the Unit still needs to be improved.

1.4 Mating Compatibility of *Anastrepha fraterculus*

Teresa Vera from Argentina spent several months in the Unit carrying out field cage mating compatibility studies on different geographic populations of *A. fraterculus*. These studies were carried to provide baseline data for any eventual SIT programme for this species. The studies revealed significant differences between the geographical populations especially as regards the time of mating. These differences lead to almost complete isolation of some of the populations when they were tested in the field cage. The results of this work will require that SIT programmes for this species take into account these geographical differences.

ENTOMOLOGY UNIT ORGANIZATIONAL CHART



2. Quality Control and Rearing Studies in Tsetse

The temperature and humidity control systems for the tsetse rearing container were completed so as to provide a satisfactory environment for tsetse rearing. A colony of G. fuscipes has now been introduced into the container and production levels are very satisfactory. All stages of the life cycle are reared in the container and a full evaluation will be completed in 2004. There would appear to be no technical reasons why this system cannot be used on a larger scale in Africa where required. The design of the TPU 3.2 rearing system for tsetse mass production was finalised and it is planned to install this system in the container in 2004. To improve opportunities for fellowship training a laboratory has been completely refurbished.

*Fifteen prototypes of the new cage design described in the **Annual Report 2002** were evaluated. These plastic cages are designed to be injection molded and consist of two symmetrical halves. Initial testing of the grid systems in 2002 was satisfactory but evaluation of the complete cage was disappointing with high mortality of flies in the cages as a result of inadequate feeding response. Two new designs are now being developed for the cages for TPU 3.2*

Further experiments have been carried out on the development of diagnostic primers for the virus that is present in the G. pallidipes colony originating from Uganda. The long-term aim of this work is to try to establish a virus-free colony of this species. The virus causes salivary gland hyperplasia and impacts negatively on reproductive performance. It was shown that many different tsetse species in the Unit carry the virus but it only appears to cause hyperplasia in the G. pallidipes colony where the majority of individuals carry the virus. Efforts are underway to develop a non-invasive assay for the virus.

The self stocking production system (SSPC) described in previous reports is designed to provide mature male pupae for emergence, sexual maturation, sterilization and release. The logistics of handling these large numbers of males during these procedures needs to be developed. As a start to this, the chilling experiments reported earlier have been extended to simulate the proposed time-line for sterilization and chilled release of males in an SIT field programme. The use of a programmable climate chamber much facilitated these experiments. Using a realistic time-line, no serious problems were encountered due to the effects of either chilling or irradiation. In 2004, use of the chilled adult release machine will be included in the evaluation.

2.1 Survival of *Glossina pallidipes* Irradiated and Exposed to Low Temperature

Investigations into the possibility of using the chilled adult release system are continuing as an alternative method to the release of sterile tsetse flies in cardboard boxes. Exposure of adult male tsetse flies to low temperature should be for as little duration as is possible so that the fitness of the released sterile flies is not unduly compromised. It is also necessary to ensure that losses are minimised during bulk irradiation of adult flies. After the previous experiments to determine responses of tsetse flies to certain temperature regimes, an attempt was made to simulate the actual logistics that may be involved in an operational field programme. In essence, male flies were chilled at 4°C, irradiated and then chilled at 4.5°C for 6hr and then returned to normal colony conditions. This protocol simulates radiation, transport and release of sterile male tsetse. Results reported in the **Annual Report 2002** gave some concerns as to the feasibility of using extended periods of chilling for tsetse when humidity could not be accurately controlled and new climate chambers were used for the tests in 2003.

2.1.1 Evaluation of the climate chambers

Two chambers in which temperature and humidity could be controlled, were evaluated, one of them was programmable. A number of survival tests were run and the results are shown in **Table 1**. Very good mean survival was recorded for all treatments except the last were the flies were chilled for 24hr. The LMS chamber gave the most variable results and was not used further. Humidity did not appear to be a significant factor affecting survival.

Table 1. Survival of unirradiated, unfed male *G. pallidipes* in different types of chambers (*non programmable LMS (L) and programmable Weiss (W)).

Treatment	# Cages	Mean # Flies/Cage ±s.d.	Mean Survival %±s.d.
Control, in 23°C, 75%r.h.	84	32.2±14.5	99.7±1.2
Cold, 4.5°C, 6h, 70%r.h (W).	65	34.5±19.3	98.5±2.9
Rapid cold, 4.5°C, 6h, 70%r.h. (W)	6	30.0±1.1	96.7±4.2
Cold, 4.5°C, 6h, 50% r.h. (W)	58	30.3±1.0	97.2±4.3
Cold, 4°C, 6h, 40%r.h. (L)	17	50.5±36.7	95.9±5.1
Cold, 4.5°C, 24h, 40%r.h. (W)	210	29.9±2.6	88.8±14.5

2.1.2 Overall survival

The Weiss chamber was used for the chilling procedure of 6hr at 4.5°C and 50%RH and the flies were irradiated with 120Gy in their holding cages. Survival was variable when scored the day after the test. Without further feeding about 50% survived 6 to 8 days post chilling. Flight activity in the cage was minimal after 1 week. Almost all of the flies died within fourteen days after removal from the low temperature chamber with very few surviving longer but

Table 2. The survival of irradiated, unfed male *G. pallidipes* exposed to cold and constant humidity for 6h.

Treatment	# Cages	Mean # Flies/Cage ± s.d.	Mean Survival % ± s.d.
Control, chilled	13	31±2	99.8±0.9
Irradiated, chilled	107	30±2	99.0±3.0
Control, not chilled	20	37±27	99.6±1.2
Irradiated, not chilled	13	30±2	100±0

incapable of flight activity. The overall survival results, on the morning following chilling, of all irradiated flies together with the control groups are shown in **Table 2**. Good overall survival was observed.

2.1.3 Influence of age and feeding status

Flies aged between 4 and 12 days were irradiated and their survival observed the day following chilling and irradiation. No effect of age on this parameter could be observed (**Table 3**). This is important in terms of field release strategies as males can be retained in the facility if releases cannot be carried out due to bad weather or aircraft malfunction. The data for feeding status is shown in **Table 4**. Again, no influence of feeding status could be seen on overnight survival of chilled irradiated males. This again is important data for developing the handling and release protocols for tsetse field projects.

Table 3. Influence of age on survival of irradiated, unfed male *G. pallidipes* after chilling.

Age (days)	# Cages	Mean # Flies/Cage ± s.d.	Mean Survival % ± s.d.
4	3	28±6	100
6	40	34±19	99.3±2.7
7	51	30±4	99.0±2.3
8	27	31±2	99.3±1.7
9	11	30±2	98.8±2.1
10	12	31±2	99.4±1.9
12	9	31±1	100

2.1.4 Bulked chilling and irradiation

For large SIT field projects it is essential that large numbers of insects be irradiated in large numbers. For tsetse it is currently envisaged that adult males will be irradiated in a chilled condition. Two types of bulk irradiation container were tested, an insulated expanded polystyrene (Styropore®) cylindrical container and a thermos flask, both of these containers fitted snugly into the radiation chamber. One replicate was carried out with ca 1500 male flies of mixed ages using the Styropore® container and 5 replicates, with ca 2200/replicate with the thermos flask. The containers were placed in the Co60 source at room temperature and irradiated with 120Gy. The flies were then placed in an incubation chamber for up to 6hr with temperature stable at 4.5°C and relative humidity fluctuating around 50%. The flies were then returned to normal colony holding conditions and apportioned into holding cages to observe survival without feeding. Survival was also recorded on the morning after return to normal colony conditions.

Table 4. Survival of irradiated, unfed male *G. pallidipes*.

Days Starvation	# Cages	Mean # Flies/Cage ± s.d.	Mean Survival % ± s.d.
1	55	33±16	98.7±3.1
2	98	30±3	99.0±1.4

Chilling and irradiation using a thermos flask had a little impact on survival, whereas chilling and irradiation in the expanded polystyrene container resulted in quite low survival rates **(Table 5)**. The overall length of survival was also much lower for flies chilled in the expanded polystyrene container. It was possible to maintain the flies in an inactive state when chilled in a thermos flask whereas the flies were active in the expanded polystyrene container by the time irradiation was completed. It was thus determined that it was necessary to use a container that allowed the low temperature environment to persist through the brief irradiation period and brief exposure to higher temperature during transportation. Due to the numbers of flies required to

completely fill the large container a full evaluation could not be carried out. This is quite a serious limitation in terms of developing the appropriate technology for an operational field programme. Generally, the survivors were active up to seven days after removal from the chilling chamber without any feeding.

Table 5. The survival of bulk irradiated, unfed male *G. pallidipes* exposed to low temperature.

Treatment	# Cages	Mean # Flies/Cage ± s.d.	Mean Survival % ± s.d.
Unirrad., chilled	14	128.5±31.5	96.9±5.0
Irrad., not chilled	2	119.5±30.4	100
Irrad., chilled, Styropore®	46	21.1±1.7	14.1±12.6
Irrad., chilled, thermos	60	179.3±46.9	83.1±17.6
Unirrad., not chilled	13	118.9±28.7	99.9±0.2

2.1.5 General observations

Through direct observation in the climate chamber it was noted that flies could still move/twitch their legs and drag themselves around at 6°C while they were completely immobile below this temperature. Exposure of flies to immobilising temperature for periods up to 24hr did not have any apparent effect on ability to feed and participation in mating activity even after a further 5 days of starvation, however there was lower sperm transfer volume compared to normal colony flies (control 1.6±0.4, chilled 0.98±0.4) but the duration of mating was similar (control 33.5±2.4min, chilled 30.5±6.4min). It still remains inexplicable that mortality in certain cages was very high. During the course of the year, a few trials were conducted to determine whether there was a common trend in mortality due to factors that could be controlled. It was concluded that deaths of flies in specific/particular cages was random, neither position of cage in the chilling chamber nor a specific cage were mortality factors. This work on chilling showed that it is possible to subject *G. pallidipes* to immobilising low temperature under simulated field conditions without adversely affecting the overall physiological quality of males. The effect of the chilled adult release mechanism on the flies will be investigated in 2004.

2.2 Tsetse physiology

In relation to collaborative work with Elliot Krafsur, who is the Principle Investigator on an NIH grant entitled “Ecology, Physiology and Genetics of Insect Vectors”, two participants on the grant from South Africa, Jaco Klok and John Terblanche (Univ. Stellenbosch), visited the Unit, from 17 March to 3 April, to establish standard ecological physiology protocols for tsetse flies, included water and thermal tolerances and metabolic rates. Three climate chambers were used and all experiments were carried out on *G. pallidipes* of different sexes, ages and feeding and pregnancy status. Work has also been continued on the identification of improved anti-coagulants for the blood diet.

2.2.1 Physiological assessments

Critical thermal limits, a dynamic method of determining the ecologically relevant temperature extremes (minima and maxima), were determined and behavioural observations made as to when the flies reached their CTMax - the onset of muscular spasms. These spasms represent a breakdown in coordinated motor function making it

impossible for the flies to escape such thermal conditions in the field and are the ecological limits of temperature tolerance for adult flies. The results are shown in **Table 6** and there were no significant differences in CTMax between the different flies ($F=2.0$; $p>0.178$). Preliminary CTMin values range from 4-5°C corresponding to the chilling temperatures reported above.

Water relations were assessed using gravimetric measurement of water loss over time at 24°C and 0% relative humidity (RH) and survival time, desiccation tolerance, and rates of water loss were determined. In addition, water vapour produced by the flies during flowthrough respirometry was determined. Compared to other insect species of similar size even newly emerged *G. pallidipes* adults proved to be quite resistant to desiccation with the unfed flies losing ca 33.7±2.9% of their initial mass before they died. Flies tested 2hr after feeding almost doubled their body mass as a result of the bloodmeal. Correcting for this increase in body mass they nevertheless still showed significant differences between unfed and fed teneral flies' maximum tolerable water loss ($F=135.3$; $p<0.0001$). Rates of water loss showed no significant relationship with starting mass, but were nevertheless significantly different between the fed and unfed flies ($F=57.2$; $p<0.0001$). Mean (±s.e.) rates of water loss in the fed flies was 0.158±0.006mg.hr^{-1} and in fed flies was 0.267±0.049g.hr^{-1} but this did not necessarily translate to the survival time of the flies which was 52.3±1.6 hours in the unfed teneral flies, while the fed flies survived for 57.0±3.4 hours. Therefore, these initial results suggest that, at least in teneral flies, less than 48hr old, the first bloodmeal does not necessarily contribute to the survival times of the flies. In both the fed and the unfed flies, despite working in a darkened acclimation chamber, diurnal patterns were visible in rates of water loss, probably accounted for by diurnal activity patterns such as those previously reported in tsetse.

Table 6. CTMax value of *G. pallidipes* flies expressed in °C.

Experimental Groups	CTMax Mean ± s.e.
Male, unfed, <12h	44.5±0.1
Male, unfed, >24h	44.5±0.1
Female, unfed, <12h	44.7±0.1
Female, unfed, <24h	44.5±0.1
Male, fed, >24h	44.3±0.1
Female, fed, > 24h	44.5±0.1

Flow through respirometry was used to measure metabolic rates in flies with a known history (feeding status, age etc.) and at four randomly changing temperatures, 15, 20, 24 and 28°C. Both these two sets of metabolic rate measurements were repeated several times. The data are shown in **Table 7** and an analysis of covariance of the metabolic rates of unfed and fed flies, with temperature as the covariate, showed that the fed flies had signifcantly higher metabolic rates than the unfed flies across increasing temperatures ($F=86.9$; $p<0.0001$). The post feeding processing of the bloodmeal therefore does have a marked effect on the metabolic rates of the flies but feeding status does not affect change in metabolic rate as temperatures change.

Table 7. Metabolic rates measured in of unfed and fed teneral *G. pallidipes* males at different temperatures.

Feeding Status	Temp °C	Metabolic rate, ml CO_2.hr^{-1} Mean ± s.e.
Unfed	**15**	0.0073 ± 0.0005
	20	0.0138 ± 0.0012
	24	0.0230 ± 0.0021
	28	0.0387 ± 0.0054
Fed	**15**	0.0254 ± 0.0050
	20	0.0397 ± 0.0050
	24	0.0689 ± 0.0101
	28	0.0764 ± 0.0081

Thermolimit respirometry (TLR), was used to investigate the changes in metabolism underlying the onset of the CTMax. Using flow through respirometry and electronic activity detection, TLR provides a real-time assessment of the metabolic processes underlying the physiological breakdown at the CTMax. The additional metabolism data underlying the onset of the CTMax adds to the ecological significance of the CTMax for tsetse flies. Tsetse responded in a very similar ways as beetles and ants. The CTMax of unfed tsetse males differed significantly from the thermolimit respirometry CTMax ($F=7.8$; $p<0.008$) but this was only only by 0.3°C. CTMax were 44.5±0.1°C and 44.2±0.2°C respectively. The maximum metabolic rates of the flies occured at 41.2±0.6°C and the temperatures at which the flies can metabolize maximally and the critical thermal maximum differ only by about 3°C. The technique proved to be effective for tsetse and will be used in future lab and field studies to determine the underlying metabolic responses of flies to extreme temperatures.

Water loss also responded positively to increases in temperature for both unfed and fed, however, ANCOVA results with temperature as the covariate indicated no significant differences in mean rates of water loss (**Table 8**) between unfed and fed flies ($F=0.04$; $p<0.84$). In addition, regression analyses of log water loss rate on log mass, and of water loss rates on metabolic rates indicated no significant relationships in neither the unfed, nor the fed flies for these parameters at 20, 24 and 28°C. The lack of a relationship between water loss and body size is most likely due to the small sample sizes of flies measured.

Table 8. Rate of water loss measured in mg H_2O. hr^{-1} of unfed and fed teneral *G. pallidipes* males at different temperatures.

Feeding Status	Temp °C	Water Loss, mg $H_2O.hr^{-1}$ Mean ± s.e.	Mass, g Mean ± s.e.
Unfed	**20**	0.1229 ± 0.0104	0.0274 ± 0.0010
	24	0.2717 ± 0.0235	
	28	0.4350 ± 0.0309	
Fed	**20**	0.0969 ± 0.0146	0.0525± 0.0017
	24	0.3316 ± 0.0430	
	28	0.6806 ± 0.0737	

Apart from the results obtained from the critical thermal limit and thermolimit respirometry experiments most of the results indicate that *G. pallidipes* is physiologically highly variable and this will have important consequences for its field ecology.

2.2.2 Anti-coagulants

Initial work on anti-coagulants for tsetse diet processing was reported earlier (**Annual Report 2002**). The initial trial with citrate was favourable, but the sample size was small. To increase confidence in the result the test was repeated with a larger sample, using 4 cages each of 64 females. **Table 9** presents the results, with no significant difference between defibrinated and citrated blood. The trial with pentasodium tripolyphosphate however was quite different; all concentrations tested were toxic to tsetse, with 100% mortality in all replicates in fewer than 30 days. The toxicity of this anti-coagulant would explain the very poor results obtained with the proprietary dried blood product Aprosan ® which utilizes it in its preparation

Table 9. Sodium citrate as anti-coagulant, pupal production per cage (64 females:16 males/cage).

Treatment	n	Mean	Variance	F	df	p
0.02M citrate	4	122.75	322.25	0.2855	1, 6	0.61
Defibrinated blood	4	129.75	364.25			

(**Annual Report 2002**). As a result other dried blood products, utilizing different anti-coagulants, are being sought to progress the work on artificial diets.

2.3 Colonies and Shipment

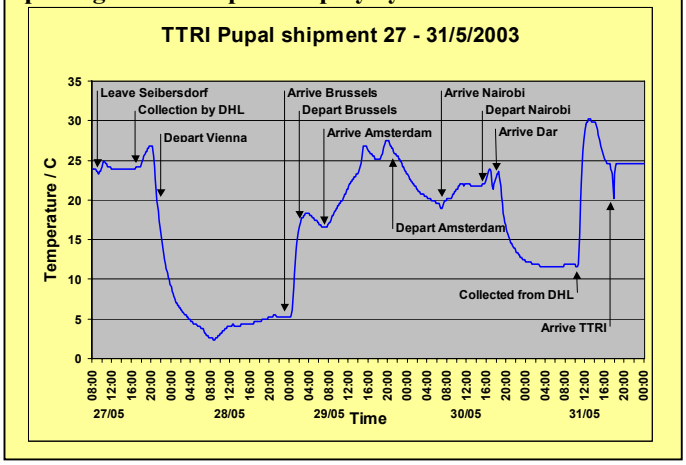
The Unit regularly ships more than twenty batches of pupae to various destinations both for research work and for colony development, with shipments totaling almost 500,000 pupae for 2003. Shipments to Africa often take an extended time, and experience both low and high temperatures in transit.

2.3 1 Pupal packaging

The emergence and survival of *G. pallidipes* pupae shipped to TTRI, Tanga, Tanzania, was below expectation through 2002 and the early part of 2003. In an attempt to improve the performance of these shipped pupae, the packaging material and the method of shipment have been improved. In addition small self-contained temperature recorders are now routinely included with the shipments.

Pupae are packaged in Petri dishes for small quantities, or in custom made perforated cardboard shipping boxes for larger quantities. The initial outer packaging was in a padded envelope. This system has proved satisfactory for the relatively short transit times involved in shipping pupae to laboratories in Europe, but was thought not to be adequate for shipment to Africa. The padded bag therefore was changed to an expanded polystyrene (Styropor®) box, 184x154x79mm internal, with a 18mm wall. The additional insulation undoubtedly assisted the pupae, but they still experience unacceptably low and high temperatures, below 10°C and above 30°C (**Figure 1**).

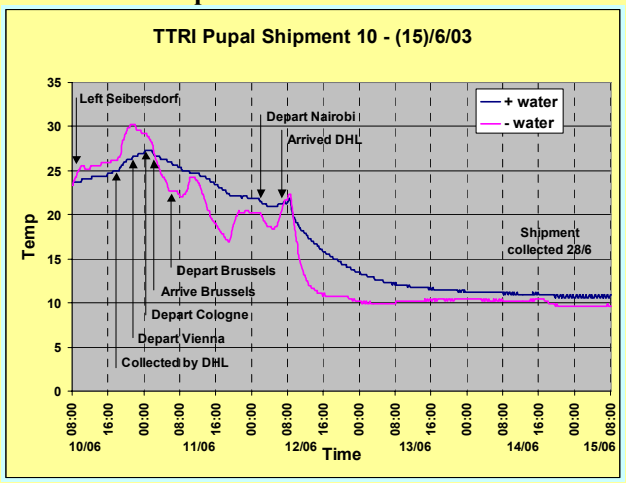
Figure 1. Temperature variation during shipment of pupae to TTRI, Tanga, May 2003. The pupae were packaged in an expanded polystyrene box.



Simple modeling of the thermal properties of the packaging indicated that the situation could be improved by increasing the total thermal bulk. The shipped pupae weigh only about 100g, so the addition of 500g of water should improve the temperature stability. For one shipment the pupae were divided between 2 expanded polystyrene boxes and two polythene bags were added, each containing 250g of water. Unfortunately this shipment was delayed in Dar es Salaam for 2 weeks, but the temperature recording over the first few days shows the effect of the addition of water (**Figure 2**). The rapid temperature changes seen without water were considerably slowed down and smoothed out, such that a given temperature drop took about 4 to 5 times longer with water than without, approximately in the proportion of the mass of pupae and of water.

Despite the addition of water as a thermal buffer the pupae are continuing to be exposed to extreme temperatures. Two further possibilities will be explored in the next year; the use of better, partial vacuum insulation, with an R value approximately 6 times that of expanded polystyrene, and the use of phase change materials for temperature stabilization.

Figure 2. Effect of adding water to a pupal shipment container on temperature variation.



2.3.2 Colony status and shipments

One of the functions performed by the Unit over many years has been to act as a stock center for colonized tsetse strains, and to supply seed material to mass rearing centres in Africa and material to researchers in various countries. The particular strains held, and the level of holding, has varied over the years according to need, but the policy is to maintain those available strains that are most likely to be used for SIT programmes in the near to mid term, but to avoid maintaining strains held at significant level by other centres.

A reduction in the staffing level in the tsetse group at the end of 2003 however has necessitated a reduction in the size of the colonies held. We have adopted the strategy of maintaining a core colony of all the strains, but reducing numbers where there is no immediate prospect of using the species for an SIT project. **Table 10** provides a summary of the strains held, the level at the start and end of 2003, and the number of pupae shipped during the year.

Table 10. Tsetse fly strains and pupal shipments, 2003.

Species	Origin of strain	Colony 01/01/03	Colony 31/12/03	Shipments 2003
G. brevipalpis	Kenya, 1987	2,473	3,842	31,710
G. f. fuscipes	CAF, 1986	25,139	6,818	179,400
G. m. morsitans	Zimbabwe	4,358	8,996	44,550
G. m. centralis	Tanzania	28,403	9,580	3,280
G. m. centralis	Botswana, 2001	437	322	0
G. pallidipes	Uganda 1975	47,941	34,010	191,450
G. p. palpalis	Nigeria 1981	5,998	4,982	43,785
G. swynnertoni	Tanzania, 1989	135	121	0
TOTALS		114,749.33	68,550.86	496,178

2.4 Rearing Systems

2.4.1 Cage development

A new cage compatible with the Tsetse Production Unit 3.2, (TPU3.2) fly holding system was described previously (**Annual Report 2002**). Problems with fly feeding through the cage grid resulted in a redesign of the grid with hexagonal holes. During 2003 this new grid design was incorporated into full prototype 300 x 300mm cages (**Figure 3**). This cage design consists of symmetric top and bottom components made

from a single mould, with a loop and catch mechanism to secure the halves at opposite corners **(Figure 4a).** The edges of the cage halves overlap alternately, and a dovetail system **(Figure 4b)** was incorporated on the sides to secure together a block of four cages to be mounted in the TPU3.2 cage holding frame. A hole to access the flies was provided at one corner, closed with a standard polypropylene cap. A matching dummy access hole at the opposite corner was also closed with a cap to improve the overall stability of the cage. The cage sample was produced by computer controlled laser stereolithography, from which a silicone rubber mould was made, from which in turn 16 half cages were cast in gray prototyping resin. Testing of the new cage prototypes however revealed that the cage grid redesign has not resolved all the problems, and the flies still do not feed well enough to support adequate reproduction. Work on cage design is continuing.

Figure 3. Plastic cage.

Figure 4. Cage detail.
a) fastening

b) dovetail

2.4.2 Tsetse rearing container

The concept of a containerized tsetse rearing system was described earlier (**Annual Report 2002**). The purpose of such a system is to address the need to rapidly establish tsetse rearing infrastructure in areas of Africa where the necessary construction skills may not be available, and to find a ready solution to the problem of a rearing facility becoming stranded behind the progressing eradication front, thereby posing a re-establishment hazard. A container can also be supplied as equipment to a project.

The concept was based on a standard, 40ft refrigerated container (reefer), adapted for the appropriate temperature range and equipped with the necessary lighting and TPU3.2. One container (without TPU3.2) was obtained for trial in the Unit, with the intention that it should eventually be transferred to a project in the field. As a first prototype a number of issues had to be resolved. Most of these were quickly dealt with, but the humidification system has proved to be difficult. In the original specification a cold, hydraulic spray humidifier inserted into the overhead ducting channel was required. These systems work well with hot-ducted air, such as the output from a furnace, but in this configuration it resulted in excessive moisture deposition inside the duct, particularly when the cooling system was running. The water lying in the duct resulted in a large overshoot of humidity at the upper set point. This has been partly resolved by removing the nozzle from the duct and providing a separate fan box, and by reducing the nozzle size, but this temporary solution whilst allowing testing to continue is not satisfactory in the long run. The container maintained its temperature

Figure 5. Blood feeding in the container.

with a daily variation of less than 0.5°C and a long term drift of less than 1°C through external temperatures ranging from –15°C up to +35°C. After the humidifier nozzle was removed from the duct, the humidity was maintained with a range of ±3%RH. To confirm the suitability of the container for tsetse rearing, a small colony of *G. f. fuscipes* has been established (**Figure 5**). To date this colony is performing well, with normal mortality (about 1%/day) and above average fecundity (greater than 0.6 pupae/female/10 days), demonstrating that the principle works. It is intended to install a full set of TPU3.2 in the container in 2004 to continue testing the concept.

2.5 Sex Ratio in the SSPC

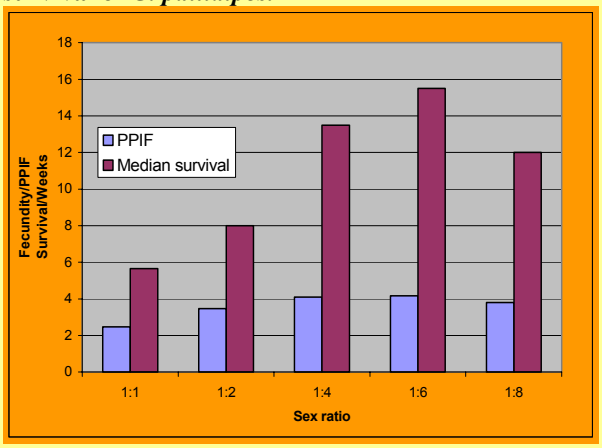
The system of sex separation and cage loading know as Self Stocking of Production Cages (SSPC) was reported previously. This system has been in use for the *G. pallidipes* colony in the Entomology Unit for several years, but a reduction in the colony fecundity together with coincidental results from other work indicated that the system might not be functioning correctly. A fellow, Gezahegn Aboset from Ethiopia, was given the task to check the performance of the SSPC system. Three cages were taken from the self-stocking system on various dates in September and October 2003 and the number of male and female flies was counted in each cage. **Table 11** gives the average number of males and females for each day, and the sex ratio. The target sex ratio for this species is between 4 and 6 females/male. As can be seen however the system failed to provide an acceptable ratio (3 or more females/male) on 14 out of 19 days, and on no occasion did the ratio exceed 4 females/male.

Table 11 Observation of the average number of female and male fly emergence in the SSPC room from 11/9/03-03/10/03.

Date	F	M	Ratio	Date	F	M	Ratio
11/9/03	62	23	2.8:1	**25/9/03**	66	31	2.0:1
15/9/03	54.3	19.3	3.1:1	**26/9/03**	61	18.3	3.3:1
16/9/03	56	24	1.9:1	**27/9/03**	60.3	26.6	2.3:1
17/9/03	69	29	2.2:1	**28/9/03**	64.6	23.6	2.7:1
18/9/03	64	28	2.1:1	**29/9/03**	62.6	43	1.8:1
19/9/03	57	26	2.1:1	**30/9/03**	57	17.6	3.2:1
20/9/03	72	24.3	2.9:1	**1/10/03**	59.3	25.6	2.7:1
21/9/03	60	13	3.9:1	**2/10/03**	61.3	30.3	2.0:1
22/9/03	55.3	37	1.8:1	**3/10/03**	64.3	17.3	3.6:1
24/9/03	68	29	2.3:1				

To follow up on this observation, sex ratios from 1:1 up to 8:1 with the *G. pallidipes* Uganda strain were tested (**Figure 6**). Median survival peaked at more than 15 weeks at a ratio of 1:6, whilst fecundity peaked at 1:4. There were marked declines of both fecundity and survival at ratios of 2:1 and 1:1; the decline in survival at 1:8 is unexplained. This clearly demonstrates the detrimental impact of resident males at ratios above about 3:1. As a result of these studies the proportion of male pupae added to the female pupae was reduced, and the system of placing pupae in emergence cages before moving to

Figure 6. Effect of sex ratio on fecundity and survival of *G. pallidipes*.

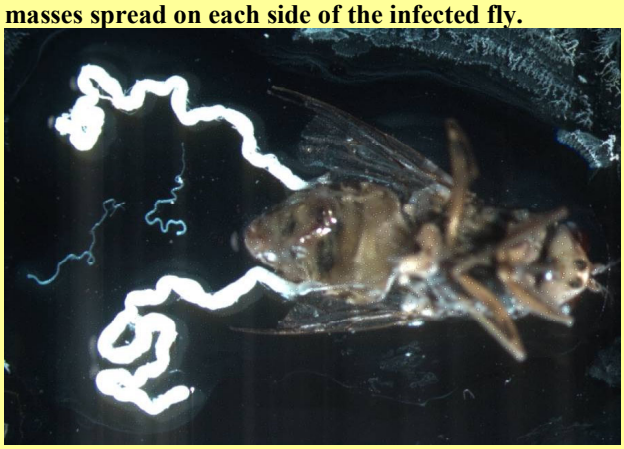


the SSPC system was dropped in favour of placing pupae directly in the SSPC system. This has resulted in some improvement in the ratios, but they remain variable resulting in sub-optimal production conditions.

2.6 Salivary Gland Hyperplasia

Viral infection associated with enlargement of the salivary glands, testicular degeneration and ovarian abnormalities was first detected in the tsetse fly *Glossina pallidipes* by Jaenson in 1978. Subsequently, similar observations have been reported for eight other tsetse subspecies. So far the virus has only been partially characterized and shown to be rod shaped with a double-stranded DNA, which appeared to be linear. In the wild, the salivary gland hypertrophy virus (SGHV) is thought to be transmitted vertically by maternal trans-ovarial transmission. The presence of the SGHV has previously been shown to affect the survival, fertility and fecundity of infected flies. Salivary gland dissections of flies from the Uganda strain held in Seibersdorf have revealed presence of the virus in the colony (**Figure 7**) but without apparent impact on the fecundity. The collapse of the Arba Minch (Ethiopia) strain a few years after its successful expansion in the Unit was nevertheless attributed to the very high infection rate observed in the colony. In view of such a precedent, it appeared important to precisely assess the status of SGHV infection in the various colonies currently maintained in the Unit. To that aim, a PCR-based procedure was designed and tested to accurately establish the epidemiological status of the colonies (**Annual Report 2002**). Herve Bossin, who joined the Unit in June, has been concentrating his efforts on the use of this very sensitive detection method for the study of the SGHV in the colonies in the Unit.

Figure 7. Salivary gland dissection of an infected *G. pallidipes*, normal transparent glands in the center left, hypertrophied salivary glands are the white masses spread on each side of the infected fly.



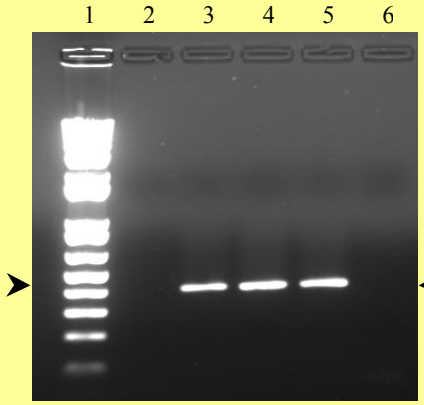
After further improvement of the PCR amplification protocol, the rate of infection as determined by patent hyperplasia was compared to that obtained from PCR analysis. In the wild, hyperplasia is seen in a few percent of flies only; Results based on PCR analysis of flies collected from Arba Minch, Ethiopia correlated well with these rare occurrences of infection (ca 2%) observed following salivary gland dissections. The PCR-based analysis, however, revealed a much higher occurrence of the virus in the Uganda *G. pallidipes* colony than initially described on the basis of salivary gland hypertrophy observations. While dissections of salivary glands revealed an average infection rate of 3.8% throughout the colony, a much higher frequency of infection was found in this same colony using the PCR method. These results reveal the extensive infection of the *G. pallidipes* colony maintained in the Unit. The asymptomatic character of this infection most likely explains the lack of significant impact on the overall colony fecundity. In view of these results, a more precise impact

assessment of the latent infection on the fecundity of the adapted *G. pallidipes* colony is being carefully analysed.

PCR analysis of the virus distribution was extended to other colonies maintained in the Unit. While the infection status for the *G. brevipalpis* and *G. fuscipes* colonies are still under investigation, the *G. m. centralis*, *G. m. morsitans*, *G. m. submorsitans* and *G. palpalis* colonies have displayed high rates of asymptomatic infection similar to those observed in *G. pallidipes*. While apparently unaffected by its presence, these colonies can act as a permanent source of SGH virus. Any introduction into the Unit of colonies potentially highly sensitive to the SGHV infection is therefore likely to result in the rapid spread of the virus and eventual loss of these colonies. The rapid collapse of the Ethiopian *G. pallidipes* colony following several generations of successful colonization could reflect such sudden epidemics likely triggered by the horizontal transfer of the virus from infected to non-infected individuals. At the time of collapse, flies from the Arba Minch colony displayed extreme levels of salivary gland hyperplasia as established by dissection. A situation never described in the field.

To better understand the causes of such widespread distribution of the SGHV in the laboratory colonies, a study of the mode of transmission of the virus under mass-rearing conditions was initiated. Particular attention was paid to the potential transfer of virus particles during the membrane feeding process. It is believed that this procedure could play an essential role in the wide dissemination of the virus between individuals and colonies. Experiments were developed where flies were artificially infected by thoracic injection of hypertrophied salivary gland homogenate. Following infection, these flies were fed on a membrane. After feeding, the remaining unused blood was collected and whole DNA was extracted from the blood. Positive SGHV PCR amplifications were obtained from the blood DNA samples (**Figure 8**), indicating the release by the artificially infected flies of viral particles into the blood on which they had been fed. While, these observations clearly show the potential role played by current mass-rearing procedures in facilitating the spread of the virus in a colony, clear evidence of a horizontal transfer is yet to be obtained. Such demonstration largely depends on the availability of a *G. pallidipes* control population totally devoid of the viral infection. Such a virus free strain is currently not available in the Unit but efforts are underway to remedy this.

Figure 8. PCR analysis of whole DNA extracted from blood contaminated following feeding of artificially infected flies.



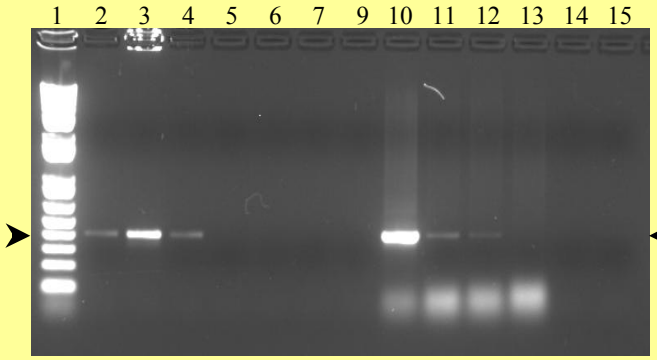
1: DNA marker
2: DNA from blood before feeding
3: DNA from blood collected after feeding of artificially infected flies (4 day post injection)
4 &5:positive controls
6: negative control.

The establishment of a virus-free colony, however, requires that uninfected flies be identified and isolated from the rest of the population at an early stage to be used to build a new colony. Current means of identification of viral infection (salivary gland

dissection, PCR analysis) in individual flies require that they be sacrificed prior to the analysis; The PCR procedure recently developed only allows for analysis of DNA samples extracted from either whole (head+thorax) flies or specific tissues (e.g. dissected salivary glands).

An investigation of alternate sources of viral template, which would leave the fly intact and available for pupae production, has recently been initiated. Tsetse meconium (substance excreted by teneral flies soon after emergence), faeces, saliva, and pupal cases have been collected and are currently being tested. Preliminary results have demonstrated that saliva collected from infected flies was a reliable source of viral particles, which can subsequently be detected by PCR (**Figure 9**). However, the collection of saliva for analysis is cumbersome and the procedures so far tested usually affect the fly viability. Techniques for non-invasive detection of the tsetse virus in individual flies are therefore still under development.

Figure 9. PCR analysis of saliva samples collected from *G. pallidipes* flies.



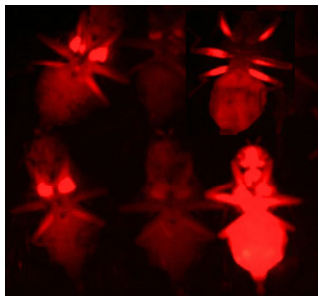
1: DNA marker, 2 & 4-9: Saliva from flies presenting normal salivary glands, 3: Saliva from fly displaying symptoms of salivary gland hyperplasia, 10-15: PCR controls, 10-12 DNA from known infected control flies, 13: DNA from uninfected control fly, 14-15: Negative controls.

Elimination of the virus from the tsetse colonies is important to prevent possible spread of viral epidemics, which could potentially threaten the invaluable colonies maintained in the Unit. While the establishment of virus-free colonies by specific selection of uninfected flies can be envisaged, a wide application of the method for replacement of all identified infected colonies does not appear sustainable without adequate parallel changes in the mass-rearing procedures. Once a virus-free colony is obtained, preventing any potential colony re-infection is vital. Application of strict quarantine rules for live field flies brought to the laboratory will have to be established and introduction of specific safeguard measures during key steps of the rearing cycle (such as artificial membrane feeding) implemented to prevent any accidental re-infection of a clean colony.

3. Genetic Sexing and Transformation in Medfly

The increased production characteristics demonstrated by genetic sexing strain (GSS) VIENNA 8 ***(Annual Report 2002)*** *have led to its adoption by most mass rearing facilities. There are currently no further GSS, based on classical genetics, under development with the exception of VIENNA 8 that carries the Sergeant mutation. Further improvements will require the induction and selection of a special type of translocation whereby the male determining factor is inserted on chromosome 5. These types of rearrangement do exist but are very rare events and they will require a large screening effort to identify suitable families. A GSS with such an insertional translocation should have full fertility.*

Customers of GSS frequently require that their local genetic background be introduced into a GSS. A relatively straightforward, but time consuming, procedure has now been developed to do this with any particular geographic population. A caveat to this is the possible occurrence of modifying genes in natural populations that interfere with the expression of the tsl mutation. A backcrossing attempt with flies from Madeira has not yet been successful, possibly because of this phenomenon. A modified protocol has now been developed and is currently being used for the Madeira population. This protocol is described and it will be completed by the end of 2004.



In the ***Annual Report 2000*** *the isolation of transformed medfly strains was described. These strains were produced using the transposable element Hermes that was isolated from Musca domestica. Two of these strains were sent to collaborators in Pavia, Italy for further study and the observations made in Pavia, especially as regards the stability of the lines, were completely different from the results in the Unit. In the Unit, the strains are stable, have a predictable phenotype and carry 1 or 2 insertions, in Pavia the lines are unstable, generate new phenotypes and carry many insertions. To try to resolve these differences further experiments have been carried out but the two sets of data remain incompatible. The reason for this is not clear and it is of some concern as stability issues are critical for any eventual use of transgenic strains in SIT.*

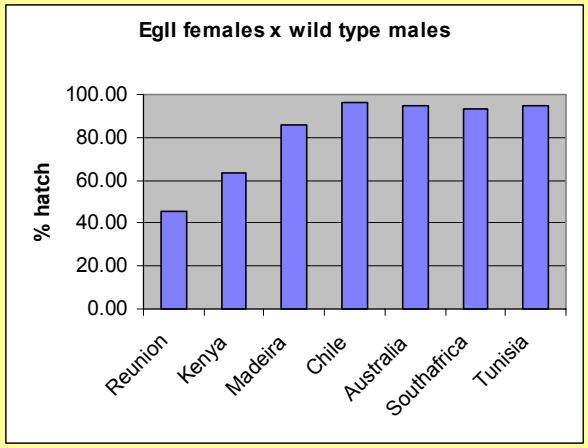
In the latter part of 2003 a new series of embryo injections was carried out using vectors that should lead to increased stability of the transgene. The use of these so-called "suicide vectors" will go some way to meeting concerns about the use of transgenic insects in SIT programmes.

3.1 Development of a GSS with Genetic Background from Madeira

All medfly GSS were initially based on a wild type strain from Egypt (EgII) that has been in the laboratory since 1963 and it has frequently been requested to introduce chromosomes from a wild type strain into a particular GSS. This is requested for two reasons: a) to increase genetic diversity by combining several wild strains and crossing them, usually in a directed manner, into the GSS, e.g. VIENNA 7/Mix 2001 with the result that a certain fraction of the genetic material in the GSS is replaced by the mixed wild type genomes and b) to completely replace the genetic material in a GSS with the genome of a population from a particular geographic area. This can be done with the exception of the two chromosomes required for sexing, i.e. chromosome 5, representing ca 16% of the entire genome, and the Y, representing ca 4% of the genome. The total replacement can only be done following many consecutive crosses with wild material whereby each cross reduces the EgII genome by 50%. In other words, a minimum of eight crosses (= 8 generations) is needed to reduce the EgII material to a level below 1%. This assumes that all combinations between wild chromosomes and EgII chromosomes are equally viable.

It has been suggested that the reduced mating efficiency of GSS males with wild Madeira females may have a genetic basis and a chromosome replacement strategy was adopted. However, earlier attempts to introduce Madeira wild type chromosomes into a GSS were unsuccessful and in crosses with other wild type strains indications of genetic incompatibility were observed. **Figure 10** shows that the egg hatch in crosses of EgII with strains from different regions, crosses involving flies from Reunion, Kenya and Madeira had reduced hatch. However in most cases problems become apparent when, after the outcrossing, the homozygous *wp tsl* strain has to be reconstructed by inbreeding. In the first step homozygosity of the fifth chromosome is monitored following the *wp* mutation. In the next step single pair crosses have to be set up and the offspring of these crosses has to be temperature tested to ensure that not only *wp* but also the *tsl* mutation is still present and homozygous. The following anomalies have been observed during the reconstruction of the homozygous *wp tsl* strain after outcrossing.

Figure 10. Egg hatch when different wild populations were crossed with EgII.



3.1.1 Guatemala wild type strain (Toliman)

After outcrossing for three generations, the inbreeding lead to severe sterility (**Table 12**). When flies from the first

Table 12. Effects of inbreeding on fertility of a medfly GSS when crossed with Guatemalan flies.

Gen	# Single Pairs	# Fertile Single Pairs	# Following Generation	# Single Pairs with *tsl*
F4	29	1	0	-
F5	26	1	0	-
F6	63	5	4	3
F7	48	22	16	6

inbreeding generation (F4) were used to set up single pairs only one of 29 pairs produced offspring and this family did not produce flies for next generation. Only from the third generation of inbreeding were fertile single pairs obtained. However, the proportion of families that carried *wp* and *tsl* decreases gradually from generation to generation presumably because of recombination.

3.1.2 Madeira wild type strain

Table 13. Outcrossing experiments with different wild type populations.

Strains	Generation	# Pairs	#(%) Fertile	#(%) *tsl*
Mad F x *tsl* M	**F1 inbred (21.1% *wp*)**	30	8	1(13)
***tsl* F x Mad M**	**F1 inbred (22.9% *wp*)**	50	14	1
	F1 inbred (24.5% *wp*)	50	28	0
	F2 inbred (15.1% *wp*)	50	30	0
	F1 inbred	150	31	0
TOTAL		330	103(31)	1(1)
Australia	**F1 inbred**	40	30	20(67)
Peru	**F1 inbred**	40	28	10(36)
Tunisia	**F1 inbred**	40	24	9(38)
South Africa	**F1 inbred**	40	33	10(30)
Chile	**F1 inbred**	40	35	30(86)
AVERAGE		40	30(75)	16(53)
***tsl* F x EgII M**	**F1 inbred (21.9% *wp*)**	50	45(90)	19(42)
Mix98 F x *tsl* M	**F1 inbred (23.6% *wp*)**	50	47(94)	1 (34)
***tsl* F x Mad M**	**Test cross**	46	46(100)	3(7)
D53 F x Mad M	**Test cross**	50	50(100)	9(18)

Initially the outcrossing was only done for one generation and here also a significant level of sterility was observed although not with the same severity as in the crosses with Guatemala material. In total 330 single pairs were set up from an outcrossing between *tsl* females and Madeira males and only 131 produced offspring **Table 13**. However, the more serious problem was that only 1 of these was *wp tsl*. The same is true for the reciprocal cross, i.e. Madeira females and *tsl* males. The results with other wild populations, EgII and a mixed population (Mix 98) are also shown in **Table 13** and between 30 and 86% of the single pair families showed the *tsl* phenotype. **Table 13** also shows that the use of the inversion D53 increases the recovery of *tsl* from 7 to 18%.

There are in principle two explanations for the phenomenon observed in the Madeira outcrossing a) Recombination: During outcrossing the *wp tsl* chromosome has to be maintained heterozygous with a wild type chromosome and in females this can lead to an unlinking of *wp* and *tsl*, i.e. resulting in the occurrence of *wp* tsl^+ chromosomes, b) Modifiers: In *Drosophila* it is known that the

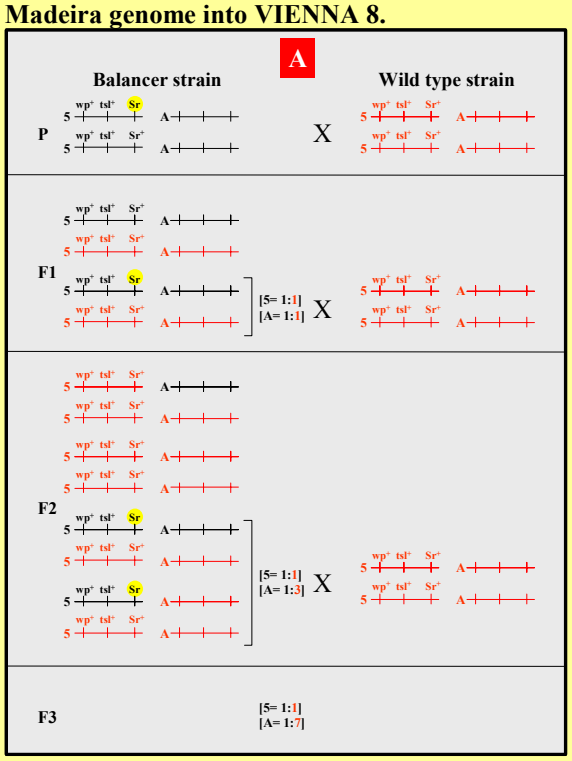
Table 14. Mating scheme for the introduction of the Madeira genome into VIENNA 8.

	A	B	%EgII
P	Ma. F x Balancer (Sr^2) M	Ma. F x T(Y;5) M	
1	F1-**A** M (Sr) x Ma. F	Ma. F x F1-**B** M	50
2	F2-**A** F (Sr) x Ma. M	Ma. F x F2-**B** M	25
3	F3-**A** F (Sr) x Ma. M	Ma. F x F3-**B** M	12.5
4	F4-**A** F (Sr) x Ma. M	Ma. F x F4-**B** M	6.25
5	F5-**A** F (Sr) x Ma. M	Ma. F x F5-**B** M	3.13
6	F6-**A** F (Sr) x Ma. M	Ma. F x F6-**B** M	1.56
7	F7-**A** F (Sr) x Ma. M	Ma. F x F7-**B** M	0.78
8	F8-**A** F (Sr) x Ma. M	Ma. F x F8-**B** M	0.39
9	F9-**A** F (Sr) x Ma. M	Ma. F x F9-**B** M	0.2
10	F10-**A** F (Sr) x Ma. M	Ma. F x F10-**B** M	0.1
11	F11-**A** F (Sr) x Ma. M	Ma. F x F11-**B** M	0.05
12	F12-**A** F (Sr) x Ma. M	Ma. F x F12-**B** M	0.025
13	F13-**A** F (Sr) x Ma. M		

Ma.=Madeira "wild" strain; Balancer=strain 68B, Sr=Sr^2; T(Y;5)=translocation strain T(Y;5)101; F=female; M=male

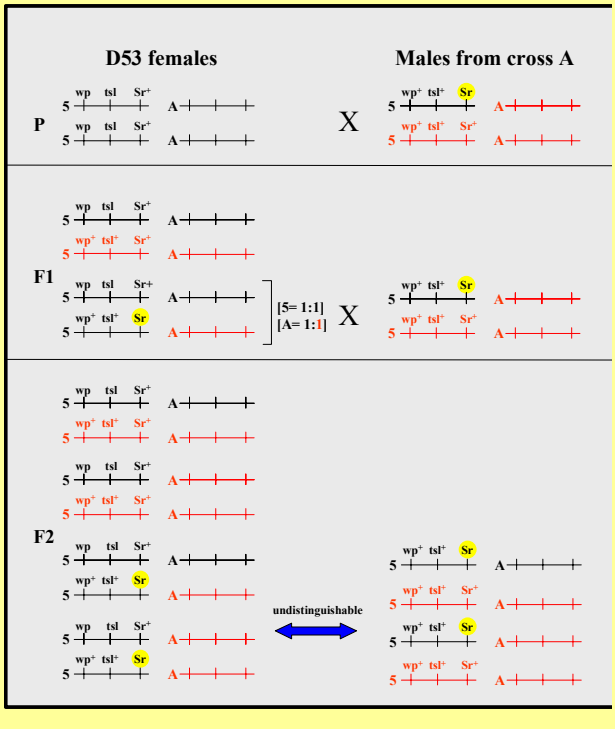
activity of genes can be suppressed or modified by sequences elsewhere in the genome. The failure to detect the *tsl* in a Madeira background could be explained by the presence of such modifiers in the Madeira genome that "inactivate the *tsl*". If this is indeed the case then there is currently no solution for this problem.

Figure 11. Genotypes involved in introducing the Madeira genome into VIENNA 8.



To try to address these issues, the crossing scheme shown in **Table 14** and **Figure 11** was developed with the aim of reducing or eliminating recombination between *wp* and *tsl* by using a balancer chromosome. Such chromosomes carry multiple, overlapping inversions and recombination in the inverted regions leads to genetically unbalanced offspring and consequently to lethality of the recombinants. Currently 13 generations of outcrossing have been completed. To achieve the level of chromosome replacement at the level required many generations of backcrossing with Madeira wild material are required. However, that leads to such a dilution of the *wp tsl* chromosome that the reconstruction of a homozygous *wp tsl* strain becomes extremely difficult if not impossible. Therefore, a dominant, homozygous lethal marker is required. Such a mutation, *Sergeant*^{2} (*Sr*^{2}), is found on the balancer chromosome. The outcrossed balancer is used to introduce Madeira wild type chromosomes into the D53 *wp tsl* strain. However, this cannot be done directly. If the crosses would be balancer-*Sr*^{2}/*Sr*$^{2+}$ x D53/D53 and in the next generation D53/balancer-*Sr*^{2} x balancer-*Sr*^{2}/*Sr*$^{2+}$, the following two genotypes would be generated: balancer-*Sr*^{2}/*Sr*$^{2+}$ and balancer-*Sr*^{2}/D53 (**Figure 12**). Both show the *Sr*^{2} phenotype and cannot be separated and this leads again to a dilution of the

Figure 12. Effect of direct crossing of D53 with non-translocated "Maderia" males.

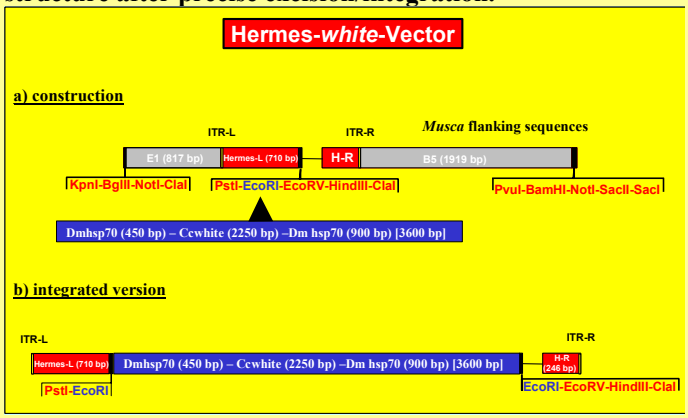


D53 chromosome during the following generations. To avoid this, the outcrossed balancer is first crossed with males carrying the T(Y;50 translocation. In parallel to the outcrossing of the balancer the translocation required for the construction of the GSS is outcrossed with Madeira material (crossing scheme B in **Table 14**). Currently we are in generation 12 of this scheme. The cross between balancer-Sr^2/Sr^{2+} females and T(Y;5) males leads to T(Y;5)/balancer-Sr^2 and T(Y;5)/Sr^{2+} males. The former males can be distinguished and are used to outcross the D53 strain. The first cross was started 12/03 with F12 females from scheme A (**Table 14**) and F11 males from scheme B (**Table 14**).

It is also desirable to maintain the mtDNA haplotype of EgII as this is very unique and can be used as internal marker for the GSS to differentiate it from wild flies. This allows the unequivocal determination of mass reared GSS flies in the field. By starting the outcrossing with D53 females and continuing with females in the following crosses the mtDNA haplotype is maintained in the selected strain.

3.2 Long term analysis of two transgenic strains

Figure 13. Construction of Hermes vector and expected structure after precise excision/integration.



In June 1998, medfly embryos from the *white* strain (*we/we*) were injected with a *Hermes-white* construct (**Figure 13)**, in collaboration with Peter Atkinson and David O'Brochta, and surviving G0 adults were mated with females of the recipient *we/we* strain. The G1 was screened for flies with red eye colour and in one cage (7-M with G0 males), 12 potential transgenics were detected (**Table 15**). These were mated individually with *we/we* flies. In one case, 7-M6F, 2 different types of non-*we/we* eye colour, red (RE) and yellow (YE), were detected. The RE and YE flies were inbred separately and were named 7-M6FRE (=6FRE) and 7-M6FYE (=6FYE), respectively. Since then the 2 strains have been reared with ca 7 pairs/generation. At each generation the flies are examined and only flies with the correct phenotype are selected. However, for several years no exceptional flies were detected.

Table 15. Results of cross of single G1 flies with *we/we*.

G1	# pupae	# RE	# WE	# YE	% emerg.	% WE	% YE	# flies
7M-1F	218	105	104		95.87	49.76		209
7M-2F	148	74	56		87.84	43.08		130
7M-3F	367	171	186		97.28	52.10		357
7M-4F	270	121	146		98.89	54.68		267
7M-5F	220	101	109		95.45	51.90		210
7M-6F	25	12	9	4	100.00	36.00	19.05	21
7M-7F	295	144	145		97.97	50.17		289
7M-1M	92	47	45		100.00	48.91		92
7M-2M	701	323	347		95.58	51.79		670
7M-3M	561	438	123		100.00	21.93		561
7M-4M	379	200	172		98.15	46.24		372
7M-5M	344	154	187		99.13	54.84		341

3.2.1 Observations on the two sublines reared in Pavia

Material of both strains (F15) was sent to Anna Malacrida, (Univ. Pavia). There the rearing was increased and the F16 was analyzed by *in situ* hybridization on mitotic chromosomes using the *white* gene as probe. The results showed that both strains contained multiple insertions many of which were heterozygous. The *in situ* analysis was repeated in F19, F29 and F34 and both, mitotic and polytene chromosomes, showed multiple insertions. During this relatively large scale rearing, flies with wild type eyes, white eyes and flies emerging from *dark pupae* (*dp*) were detected (see also below). Southern analysis (EcoRV digestion, *white* probe) showed, in addition to the endogenous white band, 2 bands in strain 6FRE while strain 6FYE was mixed, i.e. most flies showed the same 2 bands while others showed only the higher molecular weight band. It was suggested that the discrepancy between only observing two bands on Southerns and the many signals on mitotic/polytene chromosomes can be explained by assuming that each Southern band represents several independent insertions that produce, under the conditions used, the same molecular weight fragment. Sequence analyses of fragments produced by transposable element (TE) display showed, for example, that tandem integrations of the *Hermes* vector are present. Furthermore, in both strains *Hermes* elements were recovered that were flanked by at least part of the original *Musca domestica* flanking sequences, i.e. indicating the excision from the donor plasmid and the integration into the medfly genome did not occur at the inverted repeat sequences (**Figure 13**). In F24 another sample of the 2 strains was sent to Pavia. This time the material was analyzed directly. The result was again that the strain contained multiple, and in many cases heterozygous, insertions.

3.2.2 Analyses with the two strains reared in Seibersdorf

Table 16. Cytology and molecular analysis of four groups of transgenic strains.

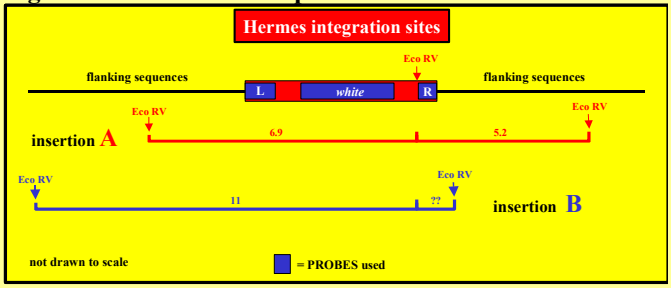
Line	Flies/1000 eggs		Chromosome Location	#	Southern	PCR[2]
	old	new				
2MRE[1]	496		4R: 57B/58A	1	Single band, ca 7kb	No
7FYE[1]	610					No
1MRE[1]	584		5L: 72B	2	Identical single unique band, >12kb.	No
1FRE[1]	261					No
2FRE[1]	392					No[4]
5MRE[1]	388					No
5FRE[1]	257					No
7FRE	492					
3FYE	599	-	6R: 97C	3	Single band, ca 7kb, similar to group 1 and to one band of group 4	-
3FRE[1]	398	598				Yes
4MRE[1]	328	284				Yes[3]
6FYE[1]	181	180				Yes
6FRE[1]	483	227	6R: 97C 5L: 65C	4	Two bands, one unique, ca 11kb, one similar to groups 1 and 3, ca 7kb	Yes[3]

[1]Analysed by Southern (EcoRV), probed with Hermes left part, i.e. without flanking and *white* sequences, [2]Primers based on inside the left part of Hermes and on the genomic sequences flanking the integration site on the left side in family 3FRE, [3]Sequence of the right integration site is identical in families 3FRE and 6FRE (i.e. presumably of the 7 kb fragment), [4] Genomic sequences flanking the insertion in family 2FRE are known to be different from those in 3FRE and 6FRE

An initial analysis of some of the *Hermes-white* lines was published and these analyses were extended (**Table 16**). The viability of the strains was determined (**Annual Report 2002**) and in F47 the insertion sites were re-examined by *in situ* on polytene chromosomes by A. Zacharopoulou (Univ. Patras). The entire *Hermes*-containing plasmid was used as a probe; this includes the *M. domestica* flanking

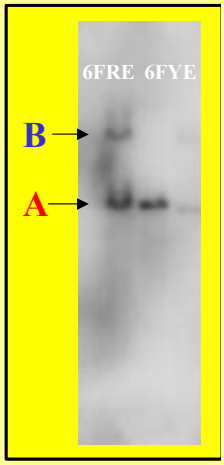
sequences. In family 6FRE, two insertions were detected, one at 97C on chromosome 6 and one at 65C on chromosome 5 (close to *white*). The latter insertion was apparently overlooked in the original analysis due to its proximity to the *white* gene. In the very few individuals that were analyzed, both insertions appear to be homozygous. The insertion at 97C is also found in the 6FYE line and in families 3FYE, 3FRE and 4MRE. **Table 16** also shows the *in situ* results of the other families. Based on the cytological location, 4 groups can be distinguished, where groups 3 and 4 have the insertion at 97C on chromosome 6 in common.

Figure 14. Restriction map of *Hermes* insertions.



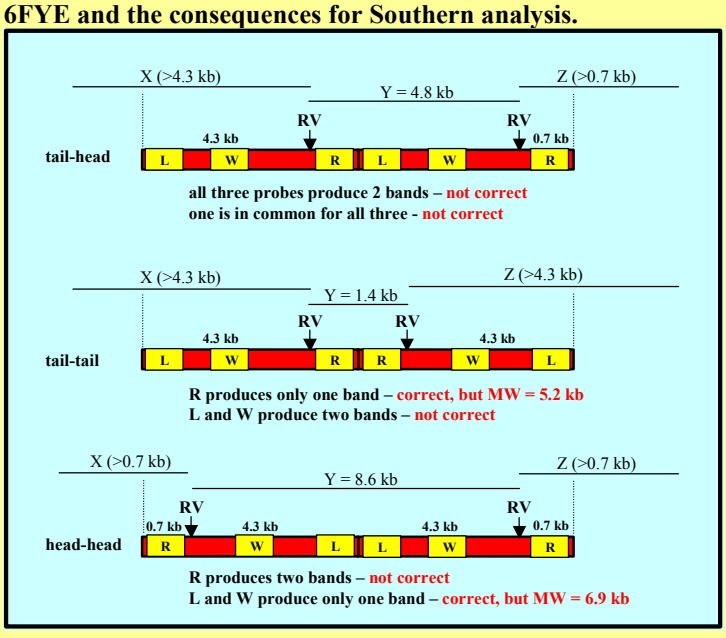
All strains were analyzed by Southern hybridization, i.e. genomic DNA was digested with EcoRV and probed with up to three different fragments internal to *Hermes-white* construct (**Table 16**). The strain 6FRE shows 2 bands on Southerns with a *white* PCR probe (in addition to the band representing the endogenous *white* gene), ca 6.9kb (A) and ca 11kb (B) in length (**Figure 14**). The same molecular weight bands are obtained with a PCR probe generated from within the left part of the *Hermes* element outside of the mini *white* gene (**Figure 15**). A probe homologous to the right end of *Hermes* generates a 5.2kb fragment, which corresponds to the A insertion. Apparently the fragment corresponding to the B insertion is missing, presumably because it is small and runs off the gel. In comparison, the strain 6FYE contains a single band of the same molecular weight as A. Because the DNA was isolated from a minimum of 5 flies, no conclusion about heterozygosity of the two insertions can be made, i.e. it cannot be determined with this method whether all flies carry both insertions and whether both are always homozygous.

Figure 15. Southern probed with *Hermes-L*.



Southerns were also performed with all the other strains and the results are in

Figure 16. Different possibilities of tandem integration in strain 6FYE and the consequences for Southern analysis.



agreement with the grouping based on the *in situ* results. Except for group 4, all strains have a single insertion.

The molecular weights as well as the number of the bands observed do not agree with a tandem integration of two *Hermes* vectors. **Figure 16** shows the theoretical results of the 3 different possibilities of a tandem arrangement for strain 6FYE. However, the Southern results with the 3 internal *Hermes* probes are not in agreement with either of these possibilities.

The conclusion that the 7kb fragment in groups 3 and 4 is indeed identical is supported by the following experiment. For family 3FRE the sequences flanking the *Hermes* element on the left and on right side are known. The sequence on the right side is identical to one of the two insertions in family 6FRE. One primer, IS-L, was designed based on the 18bp genomic sequence flanking the left side of the insertion in family 3FRE. In combination with a primer internal to the left part of *Hermes*, this primer produces a fragment of the expected size only, but in all families in groups 3 and 4 (**Table 16**). Probably due to the nature of the sequence of the flanking primer, several fragments are amplified but compared to the control a fragment with the expected size is detected only in flies with the A fragment. The empty integration site has the sequence $(AC)_6CT(AC)_2$--$(AC)_2AAAAGT$ (-- = integration site), i.e. it is probably a micro-satellite.

Additional primers were generated from the *M. domestica* flanking sequences close to either side of the integrated *Hermes* element. These were used in combination with primers internal to *Hermes*. However, none of these primer pairs gave amplification with genomic DNA from families 6FRE and 6FYE. Despite the fact that the families in group 3 carry apparently the same insertion and their phenotype varies significantly. The families 6FYE and 3FYE are pale yellow (YE), family 3FRE has red (RE) eyes but clearly lighter than wild type and family 4MRE has females with RE eyes and males that are wild type. As indicated by the name, families 3FYE and 3FRE are descendants from same G1 female. The families in group 3 also show significantly different viability (**Table 16**).

3.2.3 Status of the two strains maintained by inbreeding

3.2.3.1 Small scale rearing

Both strains have been reared for 69 generations, i.e. well over 5 years, at a level of 7 pairs per generation. The phenotype is checked every generation but no exceptional eye colours (e.g. *white* or wild type) or any other obvious mutation (e.g. *dark pupae*) were observed. In several generations, cages with 50 pairs were set up. The F1 was screened but no exceptional flies were detected (**Table 17**). Therefore one has to conclude that these strains are

Table 17. Small scale rearing of strains 6FRE and 6FYE.

Inbreeding strain 6FRE	# RE flies screened	Inbreeding strain 6FYE	# YE flies screened
F27 (50 couples)	336	**F20 (50 couples)**	295
F48 (50 couples)	617	**F27 (50 couples)**	206
F52-7 (2600 eggs)	293	**F32 (50 couples)**	145
F56-1 (50 couples)	88	**F48 (50 couples)**	617
F56-2 (50 couples)	124		
F56-3 (50 couples)	292		
F56-4 (50 couples)	339		
TOTAL	2089		1263

homozygous for the *Hermes* insertion because otherwise exceptional WE flies would be expected. Secondly, the strains seem to be stable.

3.2.3.2 Large scale rearing

It was argued that the effects observed in Pavia could only be detected if the populations are reared in large numbers. With material from F52 and F58 the rearing of the two strains was increased. It took ca 2 generations until the desired quantities (34ml of pupae for setting up of the cages, 40ml of pupae for screening) were available (i.e. in F52-3 and F58-3). In the first experiment the recoveries from strain 6FRE declined rapidly so that only 18 and 15ml of pupae were available. In total close to 17,000 and 23,000 flies were screened respectively but not a single exceptional fly was found, i.e. all flies have the correct eye colour and no other mutations were detected (**Table 18**).

Table 18. Large scale rearing 6FRE and 6FYE.

Inbreeding strain 6FRE	# RE flies screened	Inbreeding strain 6FYE	# YE flies screened
F52-3 (40ml)	1687	**F52-3 (40ml)**	2045
F52-4 (18ml)	689	**F52-4 (40ml)**	2033
F52-6 (15ml)	477	**F52-6 (37ml)**	1859
F58-3 (40ml)	2021	**F58-3 (40ml)**	2213
F58-4 (40ml)	1822	**F58-4 (40ml)**	2912
F58-5 (40ml)	1883	**F58-5 (40ml)**	2048
F58-6 (40ml)	1469	**F58-6 (40ml)**	2024
F58-7 (40ml)	1565	**F58-7 (40ml)**	2040
F58-8 (40ml)	1866	**F58-8 (40ml)**	2158
F58-9 (40ml)	1517	**F58-9 (40ml)**	2059
F58-10 (40ml)	1700	**F58-10 (40ml)**	1609
TOTAL:	16696		23000

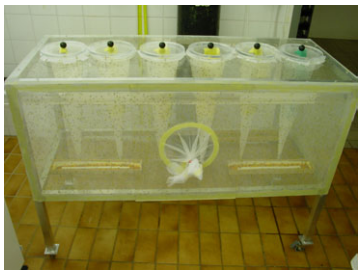
In F53 material from both Pavia strains was returned to Seibersdorf. However, only 6FYE survived the shipment. Roughly 6% showed a gray pupal colour. Later it was shown that this represents an allele of the known *dark pupae* mutation. Two parallel strains were established, 1 with wild type and 1 with gray pupal colour. These were then reared for 5 generations at a level of 34ml of pupae/generation. The majority of the flies in both strains had wild type eye colour (98.5%) and the remaining flies showed a variety of eye colours ranging from RE (0.14%), light RE (0.14%), dark YE (1.08%) to WE (0.14%).

In conclusion, it is obvious that the phenotype as well as the genotypes of the strains maintained in Pavia are clearly different from the original strains maintained continuously in the Unit. The latter are homozygous for the insertions otherwise inbreeding would result in the appearance of WE flies. The B insertion is restricted to 6FRE, i.e. unlike in the Pavia subline this insertion was never found in 6FYE. The flanking sequences at the integration site of the A insertion are as described in the original publication and are found in all families in groups 3 and 4. No *M. domestica* flanking sequences can be detected. Also a tandem arrangement of two elements can be excluded. In general, there is no obvious sign of instability despite the fact that the rearing was done for several generations at expanded level, e.g. no mutations were detected. Currently it is very difficult to explain these differences, this is especially so for the material sent as F24 to Pavia because that material was analyzed directly without any further rearing. The analyses described above deal with the two homozygous lines. Additional experiments were done to test the segregation of the insertions. The results of these tests are in agreement with the above conclusions and will be reported in the next Annual Report.

4. Fruit Fly Rearing and Quality Control

As support to the development of new strategies for the implementation of medfly SIT using GSS, studies have been carried out to identify a protocol that will enable male eggs to be shipped from a large facility, where the large production colony is held, to smaller satellite facilities where the male insects would be reared for sterilisation and release. This reduces the need to have the production colony, with all its complexity, at every facility. It has now been shown here, and elsewhere that this is a feasible strategy and it is already in use in Central America SIT programmes.

Increased resources have been directed to R and D associated with the development of the SIT for Anastrepha spp and Bactrocera oleae. In A. fraterculus, mating compatibility field tests and experiments on artificial adult and larval diet have been carried out. This species of fruit fly in present in many countries in South America and is of major agricultural importance. In the 70's and 80's much R and D was carried out on the development of SIT technology for B. oleae. The major constraints were found to be the cost of production and the change in mating behaviour induced in mass reared flies. Current activities in the Unit are focused on the evaluation of alternative dietary components for the larval diet.



In the 2001 Annual Report encouraging field cage data were presented on a genetic sexing strain containing the Sergeant (Sr^2) mutation. This is a dominant visible mutation that may be useful for field monitoring of released flies. During 2002, this mutation was backcrossed into the VIENNA 8 GSS and during 2003 the strain was mass reared to generate information on its QC protocol and on the stability of the Sergeant mutation. The results show that the inclusion of the Sr^2 mutation in this GSS did not impact negatively on the QC profile of the strain. This suggests that, together with earlier data on mating competitiveness, the strain could be further evaluated as for use in an operational SIT programme. In addition the studies on the stability of the VIENNA 8 GSS without the Sr^2 mutation were completed.

Sperm competition is important for SIT in terms of multiple mating by females. Studies carried out using the white pupae mutation have shown a marked negative effect of high doses of radiation on the competitiveness of sperm in multiple mated females. It extent to which this might impact on field effectiveness of sterile males is not clear.

4.1 Storage of Medfly Eggs

The goal of this work was to identify strategies for the preservation and transport of eggs from GSS mass rearing facilities to satellite rearing facilities where only male insects would be reared for sterilization and release. Eggs from strain VIENNA 7/Mix were exposed to different treatments for different periods of time and the effect on several QC parameters assessed.

4.1.1 Egg Incubation

Immediately after collection, the eggs were placed inside plastic bottles containing water (v/v ratio of 1:20, respectively), through which air was bubbled via an aquarium stone for 48hr. For colony maintenance eggs are bubbled for 48hr in water at 24°C. For male only production the eggs are incubated for 24hr at 24°C, followed by 12hr at 34°C and then 12hr at 24°C, completing the 48hr incubation period.

4.1.2 Storage treatments

The eggs were randomly divided into 9 groups, immediately after the first 24hr of incubation at 24°C. Each group consisted of 8ml of eggs immersed into ca 70ml of storage media. Eight out of the nine groups were stored under different combined conditions, which included two temperatures (10°C or 25°C), 3 storage periods (0, 24 and 72hr), and 2 storage substrates (sterile water or agar solution of 0.1%). The purpose of using the agar solution was to provide a more viscose media, with higher density than plain water to prevent egg sedimentation and avoid damage. The remaining group was treated as for male-only production and served as the control (**Table 19**).

Table 19. Egg treatment variables.

Group	Time (hr)	Medium	Temp (°C)
Control			
1	24	water	10
2	72	"	10
3	24	"	25
4	72	"	25
5	24	agar	10
6	72	"	10
7	24	"	25
8	72	"	25

Following these treatments the eggs were transferred into plastic bottles and treated as for male only production. Following incubation, eggs were washed with distilled water and then transferred into trays containing 5kg of larval diet. Three pupal collections/tray were made and a standard QC evaluation completed. These included; egg hatch a) immediately after egg collection, b) after the first 24hr incubation at 24°C, c) following the various storage treatments and d) following the thermal treatment at 34°C, but before transfer to larval diet; pupal emergence, flight ability and sex ratio. Dissolved oxygen (DO) or partial pressure of oxygen in the incubation media was also measured on four different occasions, following the daily egg collection, after the 24hr of incubation at 24°C and for each group, before and after the preservation under the different treatments. In addition, these parameters were also measured in incubation media without eggs.

4.1.3 Egg hatch

This parameter was significantly affected by storage time, ($F=11.41$; $P=0.002$), if storage medium and temperature were compared; the duration of the storage period

had different effects on egg hatch. For the 2 observed storage times (24 and 72hr), the hatch of eggs stored at 10°C, in water was higher for storage for 72hr than for storage for 24hr. However, eggs preserved in agar for 72hr had a lower egg hatch than their counterparts preserved in water. On the other hand, eggs stored in agar for 24hr had a higher hatch than the eggs stored under the same conditions in water. In general the results showed that egg hatch was higher for eggs stored for 72hr. However there was no statistical difference for the results obtained when all factors i.e. the type of substrate, temperature or the interaction between the 3 different factors were compared.

4.1.4 Pupal production

A summary is shown in **Table 20** and there were significant differences and in addition, the interaction of storage time, temperature and medium was significant (F=12.39; P=0.029). The 3-way interaction plot of means showed that eggs stored in agar for 24hr at 25°C produced the largest number of pupae (**Table 20**), however there was a significant difference in pupal production from eggs stored in agar or water for 72hr at 25°C. There was not a significant difference between the interactions of the other factors (**Table 20**). Based on the means, the most suitable condition for pupal production was storage in agar for 24hr at 25°C. The two-way interaction between storage medium and temperature or storage time showed that eggs stored in agar solution produced the largest number of pupae (**Table 21**).

Table 20. Adult and pupal production from medfly eggs stored under different conditions.

Variables			#	
Time	**Medium**	**Temp**	**Pupae**	**Adults**
24	**agar**	**25**	54871a	43746a
24	**water**	**25**	51431ab	42573ab
24	**agar**	**10**	52874ab	40636ab
24	**water**	**10**	50579ab	39528ab
72	**agar**	**10**	45759ab	34367ab
72	**water**	**10**	42606ab	33220ab
72	**agar**	**25**	39723b	28898b
72	**water**	**25**	16751c	12355c

Table 21. Two-factor analysis of egg storage conditions.

Variable			#
Time	**Medium**	**Temp**	**Pupae**
24	**agar**	*	53873a
24	**water**	*	51005a
72	**agar**	*	42741b
72	**water**	*	29676c
*	**agar**	**10**	49317a
*	**agar**	**25**	47297a
*	**water**	**10**	46590a
*	**water**	**25**	34091b

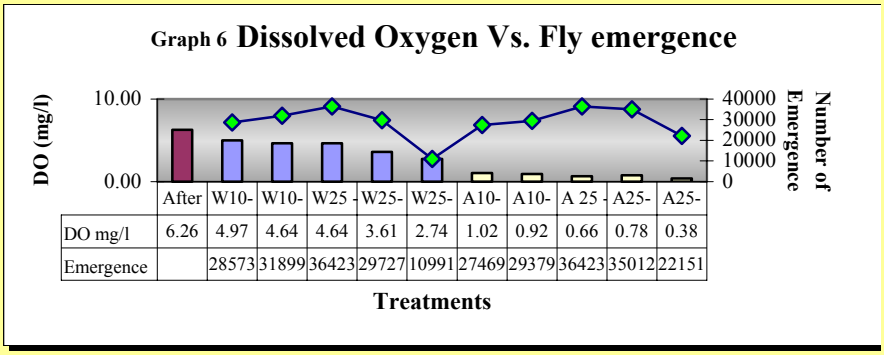
4.1.5 Adult emergence

There is a high correlation between the number of pupae and the number of adults recovered (R=0.984; P=0.00) and we concluded that any treatment effects on adult numbers were mediated through pupal production. From the 3-way analysis a similar picture emerged for pupae and adults. Although, at 10°C the longer storage period (72hr) yielded higher numbers of adults and for both times the number of adults was higher in water than in agar. At 25°C, the number of adults for the two storage periods was lower in water than in agar. In this case the 24hr storage period yielded larger numbers of adults. In general, eggs stored in agar at 25°C for 24hr provided the largest number of adults (43,746±6,046), the figure for the control group being 45,957±5,984.

4.1.6 Dissolved oxygen

There was no relationship between fly emergence or number of flyers and the concentration of dissolved oxygen in the storage media. However, a direct relation

Figure 17. Dissolved oxygen in the different egg treatment protocols. A=Agar, W=Water, CNT=Control, temp (^{0}C) – time (hr).



	After	W10-	W10-	W25	W25-	W25-	A10-	A10-	A 25	A25-	A25-
DO mg/l	6.26	4.97	4.64	4.64	3.61	2.74	1.02	0.92	0.66	0.78	0.38
Emergence		28573	31899	36423	29727	10991	27469	29379	36423	35012	22151

between the concentration of oxygen available for the different treatments and the type of storage media, temperature and storage period could be clearly observed. Lower temperatures were associated with higher oxygen concentrations. Moreover, at a given temperature, shorter storage times had higher concentrations of oxygen. The oxygen content in water was always higher. Therefore, eggs stored in water for shorter periods of time and at lower temperatures showed higher concentrations of dissolved oxygen at the end of the storage period (**Figure 17**).

These and other data have been used to develop a egg shipment protocol for the medfly programme in Central America where heat treated eggs from GSS VIENNA 7 are transported from the El Pino rearing facility in Guatemala to the rearing facility in Tapachula, Mexico and are used to rear males for sterilization and release.

4.2 Mating Compatibility between Different Populations of South America Fruit Fly, *Anastrepha fraterculus*

Clarification of the mating compatibility among geographically separated populations is important to determine the feasibility of SIT implementation on a regional basis. Previous experience in other tephritid pests, such as the medfly, has shown that rearing facilities in different Member States can use the same strain of fly in order to achieve good production and quality of flies. This has been supported by evidence that target wild populations around the world are sexually compatible with mass-reared flies. *A. fraterculus* is distributed over large areas in South America and there is no information on the mating compatibility of the different populations. In relation to the future use of the SIT for this species it was decided to evaluate the reproductive compatibility among different *A. fraterculus* populations originating from various locations within its distribution range.

4.2.1 Source of flies

The fruit fly laboratories in the Unit provide the required quarantine security to permit working with quarantine species such as *A. fraterculus*. Import permits were obtained and contacts with researchers at locations of interest were established. It was important to try to obtain wild material but laboratory colonies were also used. Colonies were established under relaxed rearing conditions (less than 7cm^2/fly and

use of fruit as oviposition substrates and larval hosts). For all the populations, specimens of both sexes were preserved in ethanol and sent to Dr. Roberto Zucchi (Univ. Sao Paulo) and Dr. Vicente Hernández-Ortiz (Instituto de Ecología, C. A., Mexico) for confirmation of taxonomic status. Pupae were received from eight locations comprising Mexico, Guatemala, Colombia, Peru, Brazil and Argentina (**Table 22).** Rearing under relaxed conditions was initiated for all the populations and only in the case of Guatemala was it not possible to establish a colony.

Table 22. Origin of the *A. fraterculus* populations.

Location	Country	Type	Name
Tapachula	Mexico	Wild	Tapachula
El Pino	Guatemala	Wild	Guatemala
Ibague	Colombia	Lab.	Colombia
Piura+La Molina	Perú	Lab.	Peru Lab
La Molina	Perú	Wild	Peru Wild
Bahia	Brazil	Wild	Bahia
Piracicaba	Brazil	Wild	Piracicaba
Concordia	Argentina	Wild	Concordia
Tucumán	Argentina	Lab.	Tucumán

4.2.2 Data analysis

In order to determine the degree of mating compatibility among populations, the Index of Sexual Isolation (*ISI*) was estimated. Its values range from -1 (complete negative assortative mating; i.e., all matings are with members of the opposite population) through 0 (random mating) to +1 (complete positive assortative mating; total mating isolation of the two populations). Together with the *ISI*, the indices of relative performance for each sex were also calculated. The Male Relative Performance Index (*MRPI*) is a relative measure of mating propensity of the males of one population versus the males of the other. It also ranges from -1 to +1. A value of 1 indicates all matings in the cage were done by the males of one population, a value of -1 indicates all matings were done by males of the other population. Zero indicates that males from both populations participated equally in mating. The Female Relative Performance Index (*FRPI*) is the counter part of the *MRPI* and serves as a measure of mating propensity for female flies.

4.2.3 Evidence of incompatibility

Twelve mating compatibility tests were performed. The sexual compatibility indices showed behavioural isolation within most of the populations analysed (**Table 23).** Piracicaba flies (both sexes) mated early in the morning while Bahia flies mated at the end of the afternoon or beginning of sunset. Therefore, no matings involving the two populations were collected and this is shown by a value of 1.00 for the *ISI*. This observation was explained by the later confirmation, from one of the reference

Table 23. The *ISI*, *MRPI*, *FRPI* for each *A. fraterculus* mating combination analysed.

Mating combination	*ISI*	*MRPI*	*FRPI*	#
Piracicaba–Bahía	10±0.0	0.37±0.37	0.37±0.37	2
Perú Wild–Concordia	0.9±0.1	-0.15±0.13	-0.11±0.09	4
Tucumán–Perú Lab	0.8±0.1	-0.14±0.09	-0.02± 0.05	4
Tucumán–Perú Wild	0.8±0.1	-0.32±0.07	-0.14±0.09	10
Perú Wild–Colombia	0.8±0.1	0.49±0.08	0.39±0.14	4
Perú Wild–Tapachula	0.8±0.1	0.24±0.12	0.12±0.08	6
Tucumán–Tapachula	0.8±0.0	0.06±0.14	0.14±0.14	6
Perú Wild–Piracicaba	0.6±0.1	0.00±0.06	-0.06±0.07	6
Piracicaba–Tapachula	0.6±0.1	0.01±0.11	0.05±0.06	6
Tucumán–Piracicaba	0.4±0.1	0.21±0.04	0.39±0.10	6
Tucumán–Concordia	0.2±0.1	-0.27±0.04	-0.18±0.17	4
Perú Wild–Perú Lab	0.1±0.1	-0.13±0.04	-0.13±0.05	4

taxonomists, that, in fact, the population from Bahia belonged to the species, *Anastrepha sororcula*. This species belongs to the *fraterculus* group, and very similar in morphology to *A. fraterculus,* yet is sexually active in the late afternoon. Since both morphological and behavioural data agreed, this population was removed from the analysis. It was also found that the flies from Colombia were sexually active at sunset. The taxonomic identification was in agreement by both specialists confirming this population to be purely *A. fraterculus*. The results are interesting since it is the first reported case in which this species was found to mate in the late afternoon. Although it was not possible to combine Colombia with all the tested populations, it is clear that this population is isolated from those that were sexually active principally during the early morning (i.e. Tucumán, Concordia, Tapachula and Piracicaba). The peak of mating activity for Peru was during the midday.

The other populations showed a peak in the beginning of the day, and this can explain the high values of the *ISI* of the 'early morning mater' populations tested against Peru. However, even within the populations that mated in the morning the degree of sexual isolation was high. In the case of Tucumán–Tapachula and Piracicaba–Tapachula, the *ISI* is above 0.5. For the case of Tucumán–Piracicaba, the *ISI* is low but close to 0.5 and, in fact, four out of the 6 replicates performed showed a statistically significant ($P<0.05$) departure from random mating (χ^2 test). These results indicate that the populations analysed are not fully mating compatible. Total mating compatibility was detected only between Tucumán and Concordia, and between the wild population and the laboratory strain from Peru. In the first case, this is in agreement with data already reported for Argentinean populations and for the second case, this is in accordance with the fact that the laboratory strain was derived from material collected some years ago in the same location where the wild material was collected in order to perform the analysis. The indices of relative performance showed, in general, that the two populations were equally active during the tests. This result strongly support the fact that the high *ISI* values found are a consequence of some incompatibility among the populations and not a side effect of one population being more sexually active than the other under the test conditions.

4.2.4 Temporal or behavioural isolation

Further testing was done to determine whether the isolation between any early morning mating population (e.g. Tucumán) and the population from Peru, which showed a midday peak, was only due to a difference in the time of day for mating activity, or it was due to some degree of sexual isolation (i.e. even in the presence of active males and females from the opposite population the flies mate with mates from their own population). In order to do so, the flies from Peru were placed in a room with a different photoperiod than the flies from Tucumán. In the first case, the lights in the room where the flies were placed turned on 4hr earlier than in the other room. Flies were again released in the field cage after sexual maturation and at sunrise. Mating couples were collected and the indices estimated. As control tests, at the same time, and with the same lots of flies, flies from Peru, but with the same light cycle as those from Tucumán, were tested against Tucumán and against the flies from Peru that had the light cycle 4hr out of phase.

The *ISI* values obtained were 0.79±0.05 (N=6), while for the control the values were 0.80±0.11 (N=4, Tucumán versus Peru normal light cycle) and 0.16±0.08 (N=4, Peru

with altered light cycle versus Peru with normal light cycle). Although it was not possible to obtain the peak of sexual activity of Tucumán flies for the Peruvian flies with the altered cycle, it was possible to move the mean time of mating closer to the normal pattern for this population (the mean time of mating start after female release was shifted from 5:46±0:16, N=17 to 3:02±0:14, N=71, while for Tucumán it was 0:58±0.07, N=100. In the test in which Tucumán flies were placed with flies from Peru with the modified light cycle, 88% of the matings involving Peru males and females took place during the range in which all the Tucumán homotypic matings took place. For the case of Tucumán versus Peru flies with the normal phase, only 53% of matings involving Peru males and females took place during the range in which all the Tucumán homotypic matings took place. The fact that in both cases the values of *ISI* were equal shows that the isolation found between the two populations has an important component of sexual or behavioural isolation and is not purely temporal.

This analysis has revealed some evidence of pre-zygotic isolation within some populations of *A. fraterculus.* This fact is of paramount importance for any future SIT programme for this species and should be confirmed in every particular case between the target population and the mass-reared flies to be released. These results support the idea that *A. fraterculus* populations are sufficiently reproductively isolated to show some degree of genetic variation resulting in different subgroups and suggest that any SIT approach should be performed with a mass-reared strain derived from the target population or from a geographically close population.

4.3 Studies on Diets for *Anastrepha fraterculus* and *Ceratitis capitata*

Improving diets for both adult and larval fruit flies remains a key activity in fruit fly SIT programmes both in terms of reducing costs of production and in terms of better quality flies. In addition it is sometimes necessary to identify and test local diet ingredients when new rearing facilities are established.

4.3.1 Adult diet for Anastrepha fraterculus

The colony of *A. fraterculus* colony established in the Unit was not highly productive because of very low egg hatchability that was thought to be due to an inadequate adult diet especially as regards the protein content. Four different adult diets were evaluated using different protein sources: Diet 1–yeast hydrolysate enzymatic (YHE):Sugar (1:3); Diet 2–corn hydrolysate protein:YHE:sugar (3:1:3); Diet 3–corn gluten meal:YHE:sugar (3:1:3) and Diet 4–olive hydrolysate protein:YHE:sugar (3:1:3). 100 virgin females and males were collected and allowed 10 days to become sexually mature during which time they were fed on the different diets. 50 flies of each sex were then put into 2 cages and individual pairs isolated that mated for at least 1hr. After separation groups of 20 females were placed in a small cage with an oviposition device. Eggs were collected daily and egg hatch measured. After 10 days the females were dissected to check for insemination. This protocol was used to ensure that all females used in the evaluation had been mated so that egg hatch would be a meaningful parameter.

The results are shown in **Table 24** and statistical analysis showed that the best adult diet for egg hatchability was the diet 2 but in addition, dissection of spermatheca

showed the highest amount of sperm and both mating frequency and duration produced the highest values. Further validation tests under semi-mass rearing conditions confirmed the above data. In a large cage stocked with 650ml of *A. fraterculus* pupae the mean egg hatchability for a 7-day sampling period was 78%. In a second cage stocked with 1L of pupae the mean egg hatchability for an 8-day sampling period was 83%. Furthermore, with minimum adjustments and an adequate larval diet the *A. fraterculus* colony can now be developed for mass rearing research.

Table 24. Effect of different adult diets on egg production and egg hatch in *A. fraterculus*.

Diet	Eggs/Female/Day	% Egg Hatch
1	4.1b	14.3b
2	16.3a	59.9a
3	14.9a	17.3b
4	9.8b	13.4b

4.3.2 Larval diets for mass rearing Anastrepha fraterculus and Ceratitis capitata

Table 25. Different diets and their ingredients tested for larvae of *A. fraterculus*.

Ingredient	1	2[2]	3[3]	4	5	6	7	8	9
Wheat. germ	6.0	3.0	3.0	0.0	0.0	8.0	2.0	0.0	0.0
Corncob	0.0	15.0	15.0	15.0	0.0	0.0	0.0	0.0	0.0
Wheat. bran	0.0	0.0	0.0	0.0	13.0	14.0	18	18.0	0.0
Cornflower	0.0	8.0	8.0	11.0	11.0	0.0	0.0	0.0	0.0
Casflower	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.0	0.0
Beetbagasse	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.0[4]
Canebagasse	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Yeast	6.0	5.9	6.0	5.0	5.0	5.0	5.0	5.0	7.0
Sugar	6.0	8.0	8.0	8.0	8.0	8.0	8.0	6.0	8.0
Na .Benzoate	0.1	0.23	0.23	0.2	0.2	0.2	0.2	0.2	0.2
Hydchl. acid	0.6	0,63	0,63	0.5	0.6	0.6	0.8	0.8	0.8
Nipagin	0.8	0.14	0.14	0.2	0.2	0.2	0.2	0.2	0.2
Agar	1.0[1]	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Water	40	59.0	59.0	60.0	62.0	64	65.8	65.8	59.8
Ingredient	**10**	**11**	**12**	**13**	**14[6]**	**15**	**16**	**17**	**18**
Wheat. germ	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Corncob	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wheat. bran	4.0	28.0	0.0	2.0	0.0	2.0	0.0	0.0	0.0
Cornflower	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Casflower	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Beetbagasse	14.0[5]	0.0	18.0	18.0	31.0	0.0	0.0	0.0	0.0
Canebagasse	0.0	0.0	0.0	0.0	0.0	18.0	18.0	18.0	18.0
Yeast	13.0	7.0	13.0	5.0	0.0	5.0	5.0	7.0	9.0
Sugar	8.0	13.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Na. Benzoate	0.2	0.28	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Hydchl. acid	0.8	1.5	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Nipagin	0.2	0.0	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Agar	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Water	59.8	50.4	60.6	65.8	60.0	65.8	67.8	65.8	64.0

[1]Agar in 200ml of boiled water; [2]Seibersdorf diet with agar; [3]Seibersdorf diet without agar; [4]Sugarbeet bagasse not blended; [5]Sugarbeet bagasse blended; [6]Seibersdorf diet for *C. capitata*

Various larval diets were tested for *A. fraterculus* (**Table 25**). Based on initial results of these 18 diets, 6 were selected for further tests using 5 replicates of trays with 250g of diet seeded with 200 eggs bubbled for 48hr placed onto a fine strip blotting paper placed on top of the diet. The egg hatch, pupal recovery and pupal size were noted for each diet and the results are shown in **Table 26**. All produced satisfactory data and one of these will be chosen to develop a large colony of this species for rearing research in 2004. The sugar beet bagasse was very satisfactory and it will probably be the bulking agent used in for the *A. fraterculus* larval rearing diet in the Unit.

In relation to the possible development of an SIT programme for medfly in Brazil, it is important to identify local sources of supply for some key diet ingredients. This can have a great impact on costs and logistics. In addition, new local ingredients have been identified in Austria that may reduce the costs of fruit fly rearing in the Unit. A consultant from Brazil, Raimundo Braga, has been helping us with these studies. A

series of different diets were compared where the new ingredients were tested and compared to the standard diet in use in the Unit (**Table 27)**. The standard diet contains wheat bran as the bulking agent but this is not available in Brazil. An alternative to this would be sugar cane bagasse but this is not available in Austria, however, sugar beet bagasse is readily available, cheap and in a pelleted form which makes storage very efficient. Soya protein is also readily available in Brazil and it is extremely cheap and it was tested as a replacement for brewers yeast.

Table 26. Larval diets for *Anastrepha fraterculus*, and effects on egg hatch, pupal recovery, pupal weight and adult emergence.

Diet	% Egg Hatch	% Pupal Recovery	Pupal Wt (mg)	% Adult Emergence
3	59.2	66.0a	16,45b	92.6
9	60.8	58.0b	13.55c	91.0
13	62.4	68.0a	17.06b	91.8
16	61.2	61.0b	14.61c	91.0
17	60.4	67.8a	17.68ab	91.4
18	56.6	62.4ab	18.43a	91.6

*Data followed by the same letter in the same column do not differ significantly according to Tukey`s HSD test (P>0.05).

In general the sugar beet bagasse was a satisfactory replacement for wheat bran but as all the QC data for medfly GSS has been developed using wheat bran it will continue to be used in the Unit. Diets 1 and 4 were compared to test the suitability of soya bean protein as a replacement for brewers yeast. 250g of each diet were seeded with 0.2ml of eggs from VIENNA 8 and the experiment was replicated 5 times and the results are shown in **Table 28**. The numbers of white and brown pupae were noted and there was no statistical difference in any of the parameters measured for the two diets.

Table 27. Different larval diets and their ingredients (%) for medfly.

Ingredients	1*	2**	3***	4	5	6	7	8
Wheat bran	28.0	4.0	4.0	28.0	28.0	28.0	28.0	0.0
Soybean protein	0.0	0.0	0.0	7.0	9.0	14.0	17.0	28.0
Sugarbeet bagasse	0.0	21.0	21.0	0.0	0.0	0.0	0.0	0.0
Brewer Yeast	7.0	7.0	7.0	0.0	0.0	0.0	0.0	0.0
Sugar	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0
Na Benzoate	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28
Hydrochloric acid	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.6
Water	50.6	53.2	53.2	50.2	48.2	46.2	43.2	57.0

* Standard Seibersdorf, ** Sugar beet bagasse blended, *** Sugar beet bagasse not blended

Table 28. Pupal production of VIENNA 8 in two types of diet.

Diet	Pupal Production (ml)	# *wp* Pupae in 5ml	# wp^+ Pupae in 5ml	Total # Pupae in 5ml	Wt(g) 100 *wp* Pupae	Wt(g) 100 wp^+ Pupae
1	35.8±0.9	126.8±4.7	144.4±6.4	271.2±5.0	0.85±0.01	0.81±0.01
4	35.0±0.6	119.2±3.6	155.0±9.5	274.8±12.7	0.82±0.01	0.80±0.01

4.4 Sperm Competition

Multiple mating of females occurs in medfly both in the laboratory and in the field. In field cages between 5-17 % of females remated and the higher values for remating are always associated with the situation that the first male to mate is the sterile male. Studies have shown that this can be correlated with both quantity of sperm and seminal fluid that is transferred during the mating. Sperm displacement can also take place in favour of the sperm from the last male but this fact can be influenced by the

overall fitness of the sperm of the strains that are in competition. All these effects can of course impact on the effectiveness of an SIT programme against this species This study reports on the effect of radiation on sperm competitiveness using the genetic marker *white pupae* (*wp*).

4.4.1 Remating with wp/wp males

Pupae from the EgII wild type strain were irradiated with 0, 60, 80, 100 and 150Gy. After 4 days of adult emergence males from each group were mated to *wp/wp* females in separate cages and around 30 mated couples were isolated where the mating time was at least 1.5hr. After separation the females were kept in individual cages and 24hr later 5 *wp/wp* males were introduced into the cage. Ten females that remated for at least 1.5hr were isolated and after separation kept in individual cages. Eggs from 10 days of production were collected and transferred to larval medium and egg hatch and the phenotype of F1 pupae were recorded.

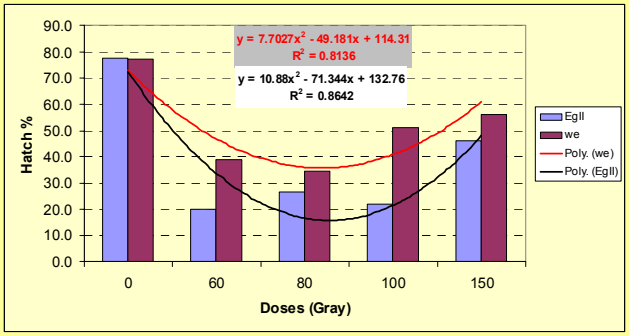
4.4.2 Remating with EgII males

A group of *wp/wp* females were confined with *wp/wp* males and around 30 mated couples were isolated where the mating time was at least 1.5hr. After separation the females were kept in individual cages and 24hr later 10 EgII males irradiated with the doses described above were introduced into each cage for 48hr. To ensure that sufficient irradiated sperm was transferred to the female 10 females that remated 3 times were separated and eggs from 10 days of production were collected and transferred to larval medium and egg hatch and the phenotype of F1 pupae were recorded.

4.4.3 Egg hatchability

Egg hatchability for all treatments showed a polynomial pattern (F=30.69; P=0.00) (**Figure 18**) with normal egg hatch being observed in females that were inseminated the first or second time by untreated EgII males (controls). Egg hatch was lowest in females mated first with an EgII male treated with 60Gy and subsequently remated with a *wp/wp* male, however egg hatch increased when the first mating was performed by EgII males irradiated with doses higher than 60Gy. Rather surprisingly egg hatch was not significantly different between the controls and from females where the first mate was an EgII male treated with 100 or 150Gy (F=13.49; P=0.00) (**Table 29**). The data in **Table 29** also shows that within a radiation dose, the hatch was consistently higher when the second male was not irradiated.

Figure 18. Egg hatch from multiple mated females.



4.4.4 Proportion of wp/wp F1 progeny

In the unirradiated control mating with EgII the second mater almost all the F1 pupae were brown indicating that there was major sperm displacement of the *wp/wp* sperm (**Table 29**). The equivalent mating sequence when the EgII male was irradiated lead to a significant increase in the proportion of *wp/wp* in the F1 progeny, indicating a major impact of radiation on sperm competitiveness (**Table 29**) In the unirradiated control mating with *wp/wp* as the second mater about 25% of F1 pupae were *wp/wp*. When irradiated EgII males were used as the first mater again there was a significant increase in proportion of *wp/wp* progeny in the F1. Using 150Gy there was a doubling in the proportion of *wp/wp* progeny in the F1.

Table 29. Egg hatch and % *wp/wp* F1 offspring in multiple mated females.

Dose	Mating Sequence		% Egg Hatch ± sd	% *wp/wp* ± sd /100 eggs
	1	2		
0	*wp/wp*	EgII	77.5±10.9a	1.4±1.3c
0	EgII	*wp/wp*	77.1±7.2a	25.3±10.2c
60	*wp/wp*	EgII	19.8±10.6b	14.0±9.7c
60	EgII	*wp/wp*	38.7±20.2b	31.1±22.1ab
80	*wp/wp*	EgII	26.5±15.5b	25.3±18.3c
80	EgII	*wp/wp*	34.5±16.9b	28.4±25.7c
100	*wp/wp*	EgII	22.1±18.6b	16.9±13.8c
100	EgII	*wp/wp*	50.9±11.9a	48.0±12.4ab
150	*wp/wp*	EgII	46.1±20.6b	32.7±21.8ab
150	EgII	*wp/wp*	56.2±21.4a	53.1±15.8a

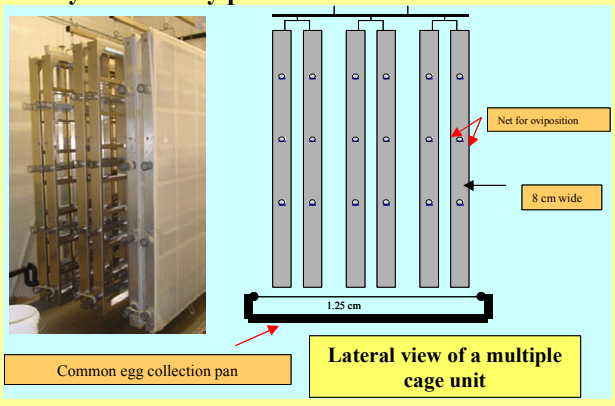
These results show that radiation does have a significant effect on sperm competition, unirradiated EgII sperm can almost completely displace *wp/wp* sperm but this effect is significantly less when the EgII males are irradiated; this despite the fact that the females were mated 3 times with the irradiated male. In fact at high doses the irradiated sperm is very poorly competitive in the female. However sperm from males irradiated with 60, 80 and 100Gy showed an acceptable degree of competition.

High irradiation doses produce high levels of sterility but low levels of sperm competitiveness. This should be taken into account when deciding on a radiation dose for released flies. This is especially relevant when males from GSS are released resulting in a very distorted sex ratio in the field that may actually increase the proportion of wild females that remate.

4.5 Evaluation of New Cage for *tsl*-Based GSS

In the previous **Annual Report**, a new cage prototype was described which aimed to address the problem of reduced egg production of *tsl* GSS in mass rearing facilities. A cage based on this design was constructed and evaluated. The new design also includes a common pan that was used to collect the eggs from the 6 cages (**Figure 19**). In a footprint of 5.5m^2 (including handling area) 6 multi

Figure 19. Photograph and schematic of new cage for medfly GSS colony production.



compartment narrow cages were set up and stocked with ca 73,000 females/cage from VIENNA 8. In parallel a conventional cage was set up with a similar density to be used as a control.

Daily egg production, the number of egg/female/day and the volume of eggs/m^2 was computed and compared with the equivalent parameters for 3 conventional cages, that being the number that can be placed in a similar area. The results have shown that egg production using the new multiple cage design can increase egg production by a factor of 2.3/equivalent floor space (**Table 30**). If the new cage design was introduced into the current medfly mass rearing facilities or used in the design of new facilities design at least half of the space used for holding the *tsl* GSS colony could be saved. The final configuration of the multiple cages design is versatile and the size and number of cages for each unit can be dependent on the facility size and design. The compartment principles used in the design of this cage would also be valid for the mass rearing of other fruit flies.

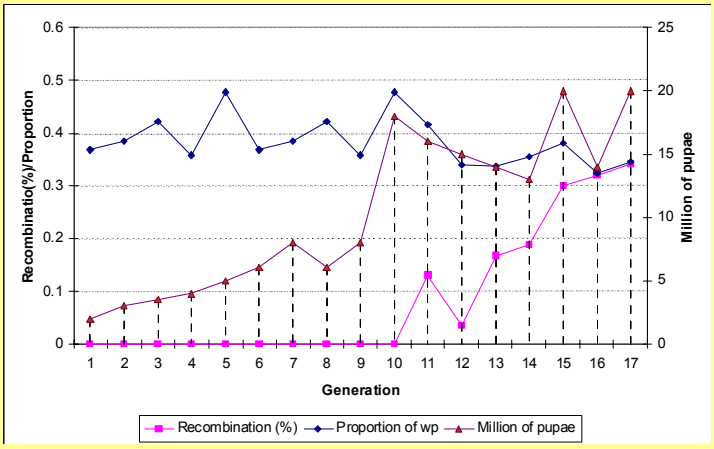
Table 30. Volume of eggs (ml) in the new and old medfly egging cages.

Days	Eggs/Day		Eggs/Female /Day		Eggs/m^2	
	New	Old	New	Old	New	Old
1	245	108	14	11	45	20
2	520	243	33	25	95	44
3	585	216	30	22	106	39
4	585	216	33	22	106	39
5	500	216	28	22	91	39
6	420	189	18	14	76	34
7	360	189	24	19	65	34
8	315	135	20	19	57	25
9	290	135	16	14	53	25
10	235	108	13	11	43	20
Mean	405.5	175.5	23.1	18.0	73.7	31.9

4.6 Stability of VIENNA 8 GSS During Mass Rearing

The VIENNA 8 GSS carries a pericentric, homozygous viable, inversion (D53) on chromosome 5 in females, designed to increase genetic stability. To verify this increased stability the strain was mass reared for 17 generations. The standard mass rearing protocols were followed to assess stability using larval trays with 5kg of diet inoculated with ca 80,000 eggs. The production colony averaged 1 million females/generation and this was able to produce an average of 10 million pupae of both sexes/generation. Rearing efficiency from egg to pupae was normal for this strain, the average being 0.43±0.09.

Figure 20. Mass rearing of GSS VIENNA 8.



The mean proportion of females (*wp/wp* pupae) was observed in each generation. The expected ratio of females to males for VIENNA 8 GSS is 0.50 but as females are also sensitive to temperature in the larval stage it is expected that there will be a slightly lower ratio due to the heat stress during mass

rearing. During the 17 generations that the strain has been maintained in mass rearing conditions the proportion of white pupae observed was 0.38±0.06 indicating that the strain was submitted to some stress during the mass rearing conditions (**Figure 20**). When type-1 recombination occurs, males are produced that are sensitive to temperature and emerge from *wp/wp* pupae and females are produced which are resistant to temperature and emerge from brown pupae. During the 17 generations in mass rearing there was no accumulation of females emerging from brown pupae indicating the high level of stability in this GSS. From generation 11, a low rate of males emerging from *wp/wp* pupae has been observed and the genetic origin of these males will be investigated in 2004.

The VIENNA 8 strain was maintained in mass rearing conditions over 16 months and only after 11 months a few recombinants were observed. For mass rearing facilities this strain would have clear advantages not just because of its increased productivity but also because it can greatly simplify the operation of the Filter Rearing System. The strain has already been provided to 4 rearing facilities during 2003.

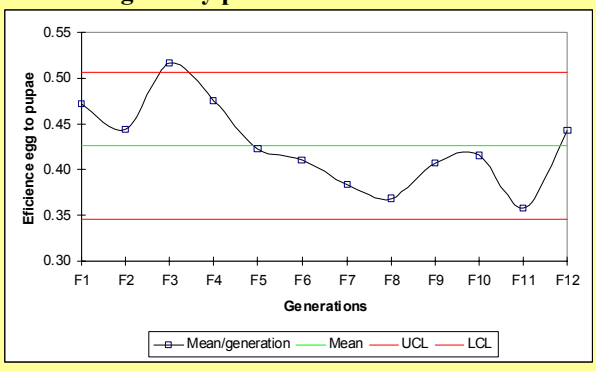
4.7 Construction of VIENNA 8 *Sergeant* and Mass Rearing Evaluation

The dominant mutation *Sergeant, Sr^2*, has been proposed as a possible visible maker for medflies released into the field. It is dominant and homozygous lethal. Initial work on the fitness of the mutation in terms of mating behaviour showed no negative effects on strains carrying the mutation (**Annual Report 2001**). It was then decided to introduce the marker into the VIENNA 8 strain and complete a full evaluation of strain during mass rearing. The mutation was combined with VIENNA 8 through a crossing scheme that relied on recombination to introduce Sr^2 onto the male linked translocation chromosome of the GSS.

4.7.1. Introduction of Sr^2 into VIENNA 8

Translocation males from VIENNA 8 were crossed to Sr^2/+ females. F1 Sr^2/+ males were selected then crossed to wild-type females and recombinant F2 males identified that carried the Sr^2 marker on the translocated chromosome. These males were then used to create VIENNA 8/Sr^2. As this crossing scheme utilized recombination to introduce the marker onto the translocation chromosome, it was possible to calculate that the recombination between the Sr^2 and the translocation breakpoint was 0.144%. This will of course have some impact when the strain is mass reared in terms of stability.

Figure 21. Rearing efficiency for VIENNA 8/Sr^2 GSS during colony production.

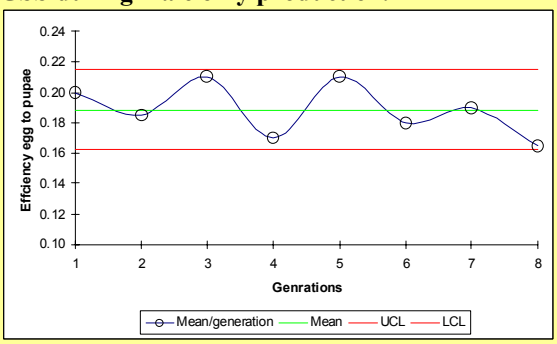


4.7.2. Mass rearing of VIENNA 8/Sr^2

The strain was evaluated under the routine mass rearing conditions in the Unit and ca 4 million pupae were produced in each generation. The efficiency of egg to pupae was used as production parameter to analyse the production stability of VIENNA 8/Sr^2 GSS. Data from

12 generations of consecutive mass rearing were analysed for both colony production and male only production and the data shown in **Figures 21** and **22**. The plotted points, each of which represents the average of measurements in each generation and the centreline (green), is the estimate of the process production mean over all the generations. The upper and lower control limits (red) are located 1 standard deviation above and below the centreline and they provide a visual means for assessing the whole production process. The control limits, which represent the amount of variation expected for the subgroup averages, are calculated using the variation within-subgroups.

Figure 22. Rearing efficiency for VIENNA 8/*Sr*^{2} GSS during male only production.



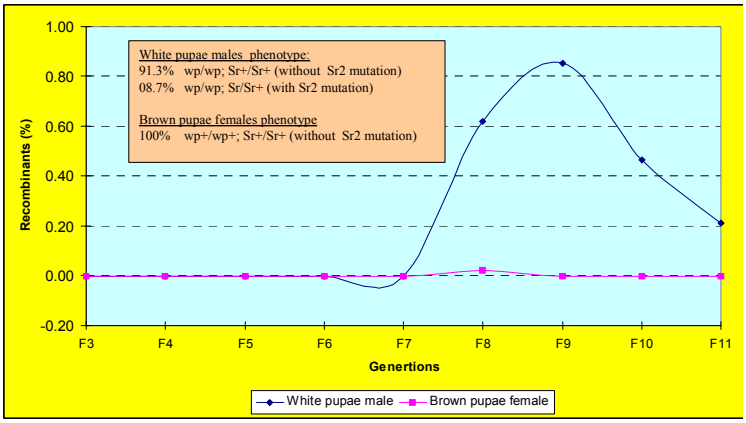
For colony production the average efficiency from egg to pupae was 0.43, which is about the average for VIENNA 8 without *Sr*^{2}. The upper and lower limits are O.51 and 0.35 respectively. For male only production the average efficiency was 0.19 with upper and lower control limits of 0.22 and 0.16 respectively. This level of male only production is also typical of VIENNA 8 without *Sr*^{2}. During the whole production process for both systems there was no deviation from the upper and control limits indicating that VIENNA 8/*Sr*^{2} shows a very stable production profile. The data for VIENNA 8 without *Sr*^{2} for rearing and quality control are discussed above and all the production statistics for the *Sr*^{2} strain are shown in **Table 31**. The two strains have very similar QC profiles and it can be concluded that *Sr*^{2} has very little, if any, impact on the ability of VIENNA 8 to be mass reared. This together with the good mating competitiveness of the strain shown in previous reports is very encouraging for its further use.

Table 31. QC profile of VIENNA 8/*Sr*^{2} GSS.

Rearing Parameter	Colony	Male only	Total
Egg hatch (%)	73.7±10.7	42.7±6.7	
Expected efficiency	0.25	0.25	0.5
Eff. egg to pupae			0.43±0.08
Eff. egg to *wp*	0.18±0.03		
Eff. egg to *wp*$^+$	0.25±0.06	0.19±0.03	
Eff. egg to female	0.15±0.05		
Eff. egg to male	0.21±0.05	0.19±0.04	
Eff. egg to flying female	0.14±0.04		
Eff. egg to flying male	0.20±0.06	0.16±0.07	
Overall % flying females	76.1±12.1		
Overall % flying males	80.1±4.3	82.0±6.9	
Flight ability index	92.8	94.7	
Sex ratio (male : female)	60:40	100:0	

During the first 7 generations of mass rearing of VIENNA 8/*Sr*^{2}, no accumulation of recombinant individuals was detected even though the Filter Rearing System was not used. From generation F8 onwards a small accumulation of males emerging from white pupae males and females emerging from brown pupae were detected, however there was no accumulation of the recombinant individuals (**Figure 23**). The penetration of the mutation is high as no males without the *Sr*^{2} mutation were detected.

Figure 23. Stability in VIENNA 8/*Sr*^{2} GSS.



These data from the characterization of VIENNA 8/*Sr*^{2} during mass rearing are very encouraging for any eventual use of the strain in an operational SIT programme. However, an open field evaluation should be conducted before any decision is made on the use of the strain. This will have to include a trapping component to assess if the marker is useful when flies are trapped in the field.

This GSS is quite complex as males carry translocation T(Y;5)101, the *Sr*^{2} marker and are heterozygous for *wp* and *tsl*, whereas females carry the homozygous inversion D53 and are homozygous for *wp* and *tsl*.

5. Mosquito SIT

The mosquito rearing facility in the Unit was officially opened in June 2003. It consists of a larval rearing room, an adult room, and office space. The insectary is equipped with the appropriate bio-safety systems including double doors and a wastewater filtration system. The adult room has a computer controlled dusk/dawn lighting system to better simulate natural mating conditions for Anopheles arabiensis. After an evaluation period where the environmental conditions were assessed a population of An. arabiensis from CDC, Atlanta was introduced and established without any significant problems. A small molecular laboratory has also been established where genetic transformation work will be carried out in relation to the development of a genetic sexing strain for this species.

The first experiments carried out were an evaluation of the two types of water that are supplied to the facility in terms of their suitability for rearing mosquito larvae. It was very quickly obvious that there was a large difference in the suitability of the water and a choice could quickly be made.

In June there was a meeting sponsored by the Agency to further plan the activities in Sudan, especially as regards the establishment of a GIS based system for the region in the Northern State where the field site is being developed. The Sudanese authorities showed strong commitment to the project and a national meeting was planned for the first months of 2004.

2004 represents the first year of the official Agency project on mosquito SIT and it was therefore possible to recruit a staff member to oversee the project. Bart Knols from the Netherlands was recruited in June. He has much experience in mosquito work in Africa and will be a very valuable addition to the Unit and a technician will be recruited in 2004. In addition Herve Bossin will join the group in 2004 to help develop transformation systems in An. arabiensis for the development of genetic sexing strains. In October, Mark Benedict had to return to CDC, Atlanta and it is planned that he will rejoin the mosquito group in 2004.

Together with colleagues from Australia and South Africa a pre-proposal was prepared and submitted to the Gates Foundation for evaluation and eventual funding.

5.1 Mosquito Strains

Initially 3 strains of *Anopheles arabiensis* were brought from CDC, Atlanta to the Unit, a wild type strain, KGB, originally colonized in Zimbabwe in 1977 and two mutant strains M2 and M5. The KGB strain was much more robust and after several months the two mutant strains were discarded. The limited number of rooms available for mosquito rearing necessitates maintaining as few strains as possible to exclude contamination. There were no problems associated with rearing the KGB strain and a thriving colony was quickly established. Until the membrane feeding was established the mosquitoes were fed on human volunteers. The adult colonies are reared in plexiglass cages with cardboard inserts to increase surface area and the larvae are fed on fish food.

5.2 Pupal density

Many parameters related to mosquito rearing efficiency will need to be assessed for developing optimal mass-rearing conditions and procedures. One of the critical parameters will be to ensure maximal adult recovery from the pupal collections. A study of the influence of pupal density on the success of adult emergence was initiated. The mosquitoes used in this study were from the KGB colony.

Pupae were collected on the day of pupation and distributed at different densities in plastic vials (9.5x2.7cm) filled with 45ml of water giving a surface area of 5.73 cm^2. For each pupal density, the numbers of emerged adults at 24, 48 and 72hr later were recorded and are presented in Table 23. The number of emerging adults was not seriously affected by the initial pupal density except at highest value.

Table 23. Effect of pupal density on adult emergence in *Anopheles arabiensis*.

# Reps	Density /vial	Density /cm^2	Time of emergence after pupation			# Adults	% Emerg.	# Pupae	♂/♀ ratio
			24hr	48hr	72hr				
50	1	0.2	0	45	0	45	90	50	ND
5	11	2	0	51	1	52	95	55	2.7:1
2	57	10	0	108	1	109	96	114	1.87:1
2	115	20	0	187	4	191	83	230	1.62:1
2	143	25	0	267	0	267	93	286	1.9:1
2	287	50	0	397	5	402	70	574	0.8:1
			0%	99%	1%				

6. Appendices

6.1 Publications

Published

ABILA P.P., M. KIENDREBEOGO, **G.N. MUTIKA, A.G. PARKER,** and **A.S. ROBINSON** (2003). The effect of age on the mating competitiveness of male *Glossina fuscipes fuscipes* and *G. palpalis palpalis*. J. Insect Sci. 3:13-19.

BENEDICT, M.Q. and **A.S. ROBINSON** (2003). The first releases of transgenic mosquitoes: An argument for the sterile insect technique. Trends in Parasit. 19:349-355.

BOSSIN H., P. FOURNIER, C. ROYER, P. BARRY, P. CÉRUTTI, S. GIMENEZ, P. COUBLE, and M. BERGOIN (2003). *Junonia coenia* densovirus-based vectors for stable transgene expression in Sf9 cells: Influence of the densovirus sequences on genomic integration. J. Virol. 77:11060-11071.

ENKERLIN, W., A. BAKRI, **C. CACERES**, J.P. CAYOL, A. DYCK, U. FELDMANN, **G. FRANZ**, **A. PARKER, A. ROBINSON**, M. VREYSEN and J. HENDRICHS (2003). Insect pest intervention using the sterile insect technique: Current status on research and on operational programs in the world. In "*Recent Trends on Sterile Insect Technique and Area-Wide Integrated Pest Management*." Research Institute for Subtropics, pp 11-24.

HENDRICHS, J. and **A.S. ROBINSON** (2003). Sterile Insect Technique. In "*Encyclopedia of Entomology*", Eds. V.H. Resh and R.T. Carde. Elsevier Science USA. pp 1074-1079.

KNOLS, B.G.J., B.N. NJIRU, R.W. MUKABANA, E.M. MATHENGE and G.F. KILLEEN (2003). Contained semi-field environments for ecological studies on transgenic African malaria vectors: Benefits and constraints. In: "*Ecological aspects for application of genetically modified mosquitoes"*. (W. Takken & T.W. Scott, eds.). Chapter 8, pp 91-106. Kluwer Academic Publishers. Frontis series no. 2.

KNOLS, B.G.J. and T.W. SCOTT (2003). Ecological challenges concerning the use of genetically-modified mosquitoes for disease control: synthesis and future perspectives. In: "*Ecological aspects for application of genetically modified mosquitoes"*. (W. Takken & T.W. Scott, eds.). Chapter 18, pp 235-242. Kluwer Academic Publishers. Frontis series no. 2.

SCHOLTE, E-J., B.N. NJIRU, R.C. SMALLEGANGE, W. TAKKEN and **B.G.J. KNOLS** (2003). Infection of adult malaria (*Anopheles gambiae s.s.*) and filariasis (*Culex quinquefasciatus*) vectors with the entomopathogenic fungus *Metarhizium anisopliae*. Malaria Journal 2:29.

In Press

CACERES, C., J.P. CAYOL, W. ENKERLIN**, G. FRANZ**, J. HENDRICHS and **A.S. ROBINSON**. Comparison of Mediterranean fruit fly bisexual and genetic sexing strains: Development, evaluation and economics. Proceedings of SAF meeting.

NIYAZI, N., **C. CACERES**, A. DELPRAT, **V. WORNOAYPORN,** E. RAMIREZ SANTOS, **G. FRANZ** and **A.S. ROBINSON**. Genetics and mating competitiveness of *Ceratitis capitata* (Diptera: Tephritidae) strains carrying the marker *Sergeant*, Sr^2. Ann. Entomol. Soc. Am.

ROBINSON, A.S, G. FRANZ and P.W. ATKINSON. Insect transgenesis and its potential role in agriculture and human health. Insect Biochem. and Mol. Biol.

SUTANTAWONG, M., W. ORANKANOK, W.R. ENKERLIN, **V. WORNOAYPORN** and **C. CACERES**. The sterile insect technique for control of the Oriental fruit fly, *Bactrocera dorsalis* (Hendel) in mango orchards of Ratchaburi Province, Thailand. Proceedings of SAF meeting.

VREYSEN, V, **A.S. ROBINSON**, J, HENDRICHS and U. FELDMANN. Activities of the Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture in support of New and Old World Screwworm eradication programmes.

6.2 Travel

Staff Member	Destination	Period	Purpose of Travel
A. Robinson	Bratislava, SLR	18 Feb	Technical assistance to IZ-SAS
	Bratislava, SLR	5 March	Technical assistance to IZ-SAS
	Bratislava, SLR	2 April	Technical assistance to IZ-SAS
	Ouagadougou, BKF	13–17 April	Installation of DNA lab, RAF5.051
	Patras, GRE	15–16 May	Invited seminar Graduate course.
	Bratislava, SLR	28 May	Technical assistance to IZ-SAS
	Edmonton and Osoyoos, CAN; Yakima, USA	21 June–4 July	Sci. Secretary of RCM D4.20.05, visit codling moth labs
	Bratislava, SLR	23 July	Technical assistance to IZ-SAS
	Kluang, MAL & Bogor, INS	20–30 Sept	Sci. Secretary of RCM D4.20.09 and invite lecture
	Tapachula, MEX	2–5 Dec	Development of a genetic sexing system for *Anastrepha ludens*. .
	Rome, ITA	8-12 Dec	FAO Expert Working Group:
M. Benedict	Khartoum, SUD and Cairo, EGY	23 Jan–14 Feb	Planning of mosquito SIT project, RAF5.052.
	Reunion, FRA	10–16 Mar	Planning of mosquito SIT project.
	Paris, Montpellier, FRA	24–27 Mar	Planning of mosquito SIT project
	San Jose (LA), USA	10–16 May	Meeting on Transg. and Genomics of Invertebrate Organisms.
	Atlanta, USA	6–12 July	Discussions malaria.
	Chania, GRE	13–19 Aug	EMBO Mosquito Workshop
	Washington, DC, USA	9–14 Sept	Sci. Adv. Com. Mtg., malaria.
C. Caceres	Bahia, BRA	13–17 January	Facility design advice
	GUA, MEX, PER	14–28 March	Deliver new GSS, advise on rearing and facility design.
	Perth, AUL	19–23 May	Sci. Secretary of RCM D4.10.16
A. Parker	Bratislava, SLR	5 March	Technical assistance to IZ-SAS
	Bratislava, SLR	2 April	Technical assistance to IZ-SAS
	Bratislava, SLR	30 April	Technical assistance to IZ-SAS
	Ouagadougou, BKF	19–23 May	Review RAF-5.051
	Bratislava, SLR	28 May	Technical assistance to IZ-SAS
	Bratislava, SLR	25 June	Technical assistance to IZ-SAS
	Bratislava, SLR	23 July	Technical assistance to IZ-SAS
	Bratislava, SLR	20 August	Technical assistance to IZ-SAS
	Montpellier, FRA	15–25 Sept	Sci. Secretary RCM D4.20.10 and IOBC-AMRQC meeting.
	Pretoria, SAF	28 Sept–4 Oct	ISCTRC meeting.
	Bratislava, SLR	15 Oct	Technical assistance to IZ-SAS
	Bratislava, SLR	14 Nov	Technical assistance to IZ-SAS
	Bratislava, SLR	10 Dec	Technical assistance to IZ-SAS
B. Knols	Bellinzona, SWI	3–6 Sept	Soc. of Vector Ecology Congress
	Dar es Salaam, TAN and Amsterdam, NET	29 Oct–14 Nov	Visit Ifakara Health R&D Centre and collaborative project
	Philadelphia, USA	3–7 Dec	52nd Meeting of the Am. Soc. of Trop. Med. and Hygiene
M. Taher	Bratislava, SLR	20 August	Technical assistance to IZ-SAS
T. Vera	Perth, AUL	19–23 May	RCM D4.10.16.
V. Wornoayporn	Bratislava, SLR	14 Nov	Technical assistance to IZ-SAS

6.3 Fellows

NAME	FIELD	PERIOD
MASHENGA, Mr. G.R.	Tsetse	2002-08-05 - 2003-08-29
BAYALA, Mr. E.	Tsetse	2002-08-19 - 2003-02-18
DEMBELE, Mr. K.I.	Tsetse	2002-10-28 - 2003-04-26
PODA, Mr. A.B.	Tsetse	2002-10-28 - 2003-04-26
DONOSO RIFFO, Mr. H.F.	Fruit fly	2003-02-03 - 2003-04-30
MOHAMED, Mr. I.A.	Tsetse	2003-02-04 - 2003-08-03
MOHAMED, Mr. Y.O.	Tsetse	2003-02-04 - 2003-08-03
MUSIE, Mr. K.	Tsetse	2003-04-20 - 2003-10-19
MODO, Mr. S.	Tsetse	2003-04-28 - 2003-10-27
DAVIDS, Ms. J.A.	Tsetse	2003-06-01 - 2003-08-31
MEZA HERNANDEZ, Mr. J.S.	Fruit fly	2003-06-16 - 2003-09-30
GEZAHEGN, Mr. A.	Tsetse	2003-07-27 - 2004-01-26
ZAMBONI COSTA, Ms. M.D.L.	Fruit fly	2003-08-11 - 2003-11-10
PARANHOS, Ms. B.	Fruit fly	2003-08-11 - 2003-11-10
HERNANDEZ ORTIZ, Mr. E.	Fruit fly	2003-09-03 - 2003-09-15
MESSI, Mr. J.	Mosquito	2003-09-08 - 2003-10-10
ZEPEDA CISNEROS, Ms. C.	Fruit fly	2003-09-15 - 2003-09-26
OUATTARA, Mr. S.J.E.	Tsetse	2003-10-06 - 2004-04-02
SAMOURA, Mr. O.	Tsetse	2003-10-06 - 2004-04-02
LUBAZIBWA, Mr. K.B.	Tsetse	2003-10-12 - 2004-01-11

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