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Genetic sexing strains in medfly, *Ceratitis capitata*, sterile insect technique programmes

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Abstract

The introduction of genetic sexing strains (GSS) into medfly, *Ceratitis capitata* (Wiedemann), sterile insect technique (SIT) programmes started in 1994 and it was accompanied by extensive evaluation of the strains both in field cages and in open field situations. Two male-linked translocation systems, one based on pupal colour, *wp*, and the other based on temperature sensitivity, *tsl*, have been used in medfly SIT programmes and they have quite different impacts on mass rearing strategy. In strains based on *tsl*, female zygotes are killed using high temperature and for *wp* strains, female and male pupae are separated based on their colour. In all these systems the colony females are homozygous for the mutation requiring that the mutation is not too deleterious and the males are also semi-sterile due to the presence of a male-linked translocation. Managing strain stability during large-scale mass rearing has presented some problems that have been essentially solved by selecting particular translocations for GSS and by the introduction of a filter rearing system (FRS). The FRS operates by removing from the colony any recombinant individuals that threaten the integrity of the strain. The use of GSS opens up the possibility of using the SIT for suppression as opposed to eradication and different radiation strategies can be considered. Some of the many field trials of the strains that were carried out before the strains were introduced into operational programmes are reviewed and an overview is given of their current use.

Introduction

Andrewartha and Birch (1960) first suggested that the efficiency of the sterile insect technique (SIT) could be improved if only sterile male insects were released and Whitten (1969) was the first to propose that genetic sexing strains (GSS) could be developed using male-linked translocations. In the sheep blowfly, *Lucilia cuprina*, several potential GSS strains were generated using a pupal colour mutation and one strain was actually used in a small SIT field trial in 1972–1973 (Whitten & Foster, unpub. data). In mosquitoes, any use of the SIT was predicated on the availability of methods by which female vectors could be excluded from the released sterile males. This essential requirement generated many studies and in several species significant progress was made (Curtis, Akiyama & Davidson, 1976; Curtis 1978; Seawright et al., 1978; Robinson, 1986). These early studies paved the way for the development of GSS in the medfly, *Ceratitis capitata*, and their eventual use in many operational SIT programmes (Robinson, Franz & Fisher, 1999). Prior to their introduction into the programmes, they had to satisfy programme managers that indeed they would lead to an improvement in overall efficiency. The fact that GSS were a new type of strain carrying different sorts of mutation was also of some concern. Three issues had to be dealt with:

(a) *GSS are descendants from a single individual carrying a unique irradiated translocated chromosome.* The concern was related to the limited genetic background and hence possible effects on field fitness. However, as with many types of specially selected strains it is possible to introduce genetic material following established

crossing procedures. Franz et al. (1996) were able to introgress a Guatemalan background into a GSS before it was introduced into a rearing facility in that country. More recently GSS have been constructed which carry components of several different genetic backgrounds (Franz, pers. comm.). Although it is logical to assume that genetic variability would be expected to lead to increased effectiveness in the field there is no data available to support this.

(b) *The increased efficiency of an all-male release in comparison with a bisexual release.* This was a key advantage projected when GSS were introduced into operational programmes but convincing field data from large-scale field tests was required. Such field trials are expensive and not always easy to implement to ensure that adequate controls are included. Rendon et al. (2000) have carried out an extensive series of these field trials at a level which provides SIT managers the answers to their questions. Rendon and colleagues were able to show quite clearly that an all-male release introduced 3–5 times more sterility into a field population than when the same number of males were released together with females. This is very convincing evidence for the use of GSS as sterility induction in the wild females is what drives the success of SIT.

(c) *Mating compatibility of GSS with geographically different populations of medfly.* The use of a proven GSS for different SIT programmes is advantageous as it would (a) enable one programme to provide sterile males to another, (b) reduce the need to provide a different strain for each facility and (c) encourage the commercialization of SIT. To investigate this point an extensive series of field cage tests was carried out in which males from GSS competed against wild males for wild females from different wild populations (Cayol, 2000). The wild populations were collected from regions representing the current distribution of the medfly. In these field cage tests using host trees, there was no evidence that there were any premating isolation barriers between GSS and medfly populations world-wide. These data support the concept of multiple use of a particular proven strain and should remove the need to cross specific genetic backgrounds into strains destined for use in particular geographic areas. Unfortunately the latter procedure is still being requested despite any evidence of its efficacy.

Two sexing systems based on male-linked translocations have been evaluated in medfly SIT field programmes, one using a pupal colour mutation, *white pupae* (*wp*) and the second using a temperature sensitive lethal mutation (*tsl*). The details of their development, field evaluation and use have been extensively described (Robinson, Franz & Fisher, 1999). This chapter will update the situation and review some of the experiences learned.

The sexing system and its operational use

Releasing only males in large-scale SIT programmes can be accomplished by either killing female zygotes at some stage of their development or selectively removing them from the population before release. These two options have very different consequences for both the logistics of mass rearing and its efficiency. Some of these consequences were predictable and others became apparent during implementation. Conditional systems that kill females have the advantage that they can be applied at the population level for example, 5 million eggs from a *tsl* GSS can be heat treated in 5 l of water. This can be contrasted with the use a 40 channel pupal colour separator which can sex only 12 million pupae/hour. The extra pupal handling, for each individual pupa can also have a negative effect on the quality of the fly.

A female killing system however, requires that two colonies have to be operated in a rearing facility. Firstly a production colony to which the restrictive treatment is not applied so that females are not killed and the colony can be maintained and secondly a release colony to which the restrictive constraint is applied to produce males for sterilization and release. The production colony must produce sufficient eggs for its own maintenance and in addition sufficient eggs to generate the release colony for male only production. For *tsl* based strains this means that in a facility there are two larval populations, one which has been heat treated and will produce only males and the second which has not been treated and which will produce males and females for colony maintenance. For systems based on selection, the sex separation can be applied on the same population that is used for colony maintenance as females can be returned after selection for mating and egg production.

It is desirable from an economic point of view that the sexing procedure, either killing or selection takes place as early in the development cycle of the insect

as possible. In medfly this has been achieved by the use of the *tsl* mutation. However the strong maternal effect associated with this mutation requires that the restrictive condition not be applied during the first 24 h of embryonic development (Fisher, 1998), otherwise the males will be killed. In the silkworm, *Bombyx mori*, a selective sexing system was developed using an egg colour mutation (Tazima, Harada & Ohata, 1951).

In general, systems based on conditional lethality in females require the application of either a chemical or a physical constraint. There are several reasons why a chemical constraint is not optimal. It is not always possible to treat developing embryos in the egg, as the chorion can be very impermeable. If a later larval stage has to be treated in the diet then relatively large amounts of chemical need to be evenly mixed with a large volume of diet. Larval diet for medfly is itself a microcosm of microbial activity which can impact on the required biological activity of the chemical. In addition, worker safety and larval diet disposal can pose problems. In medfly, two different chemical killing systems have been studied (Saul, 1982; Robinson, Riva & Zapater, 1986) but neither has been evaluated at any meaningful scale. The success in the use of the *tsl* mutation for genetic sexing in medfly is directly attributable to the fact that embryos 24–48 h old can be easily treated in a water bath. A temperature sensitive mutation that was only expressed at a later developmental stage would present serious problems for operational use, as it is not easy to regulate the temperature of large volumes of diet in large rooms.

All GSS strains based on male-linked translocations and mutations require that the females be homozygous for the recessive mutation. This places some restriction on the type of mutation that can be used. For routine laboratory maintenance of these strains, a reduction in fitness of the females is not a serious problem, however, in large facilities where economies of production play a major role in decision making, a GSS in which the females had very poor fitness would be unacceptable. All recessive mutations will be detrimental in some way or other to the female, the key question is by how much? For example reduced egg production in *tsl* GSS females requires that a proportionally larger colony is required than would be the case with a normal strain. The males in GSS are heterozygous for the mutation and would not be expected to show any detrimental effects. They do however carry a translocation that reduces the fertility of their female mates by 50%. This had to be taken into consideration during the design of the mass rearing facility.

Quality control (QC) protocols for mass produced medflies are a very important component of SIT programmes and GSS are also subject to these strict QC guidelines. Many of these protocols are aimed at pupal viability and emergence of flying males from pupae and the QC data from GSS strains sometimes gives cause for concern. The semi-sterility of translocations and hence GSS is based on the segregation of unbalanced gametes from the translocation males. These gametes carry duplications and deficiencies, the sizes of which are correlated with the position of the translocation breakpoint on the autosome and the larger the duplication/deficiency the earlier during development the zygote dies. In some GSS, duplication/deficiency zygotes are produced which survive until the pupal or adult stage. The survival of these individuals to these stages can have the effect of reducing the quality profile of the strain and indeed they make no contribution to the programme as they are of very low overall fitness.

Managing stability during mass rearing

As discussed elsewhere in this volume the problem of strain stability has to be solved before operational SIT programmes take up GSS. Much has been done to accomplish this by detailed studies on genetic recombination in GSS males, detailed mapping of mutations and translocation breakpoints and by analysis of the results of mass rearing of the strains. Using all this information, remarkable progress has been made considering the inherent variability present in biological systems. There is a point however past which improvements at the basic level of strain construction are unlikely to be made and a second level of control is required in order to maintain stability over many generations and for billions of individuals. The introduction of the filter rearing system (FRS) into GSS mass rearing provides that second level of control (Fisher & Caceres, 2000). The FRS deals with any unwanted random genetic events by removing individuals carrying them from a population. These individuals are removed by having in place a procedure whereby individual flies are phenotypically checked and, when necessary, discarded. In this way a GSS can be stabilized over time and this population is then used to produce eggs from which the males to be released are derived following amplification through

several generations. No material that has been through the amplification procedure is re-introduced into the FRS population.

A second aspect relating to the stability of mass reared GSS concerns the mass rearing procedure itself. If recombination occurs in a GSS then recombinant types are generated which have different biological fitness levels during mass rearing. By selecting particular cohorts of individuals with which each new generation is set up, the build-up of recombinants can be slowed down and stability increased. Mass rearing a GSS will always require a more careful and pro-active management approach than mass rearing a bisexual strain. Nevertheless, it is now possible to obtain 99% male medflies in routine mass rearing in operational programmes.

Prevention, suppression or eradication using GSS

The use of the SIT has traditionally been viewed as a method to eradicate local populations of pest insects using the areawide approach. This view is rapidly changing partly due to the possibility of releasing only males. In California a large aerial release programme is being carried out to prevent the establishment of medfly in the State (Dowell et al., 2000). This will be a permanent programme and therefore running costs become an important issue. The programme purchases sterile pupae from various sources and only emerges and releases the flies. Currently, releases are carried out with both bisexual and all-male sterile flies. The economics of emerging and releasing only male flies have encouraged the programme to expand its range by reducing the cost of treatment/unit area. In the future, the use of bisexual releases will be phased out (Minyard, pers. comm.).

In some areas where high quality fruit is commercially produced, the SIT, using a bisexual strain, cannot be carried out because of the damage done to certain fruit varieties by ovipositing sterile females and reliance is placed on the use of bait sprays. In these areas a switch to the use of the SIT for medfly control required a solution to the problem of the release of sterile females and a reduction in the cost of the technique. The use of GSS meets these two requirements and will lead to the use of SIT as a control technology and as a replacement for insecticidal bait sprays. The use of SIT for suppression will provide the right environment for the entry of the private sector into this field.

The appropriate radiation dose for males from a GSS

GSS are constructed using male-linked translocations and the males have a reduced fertility compared to a bisexual strain, females are fully fertile. The sterility of the male is expressed partly by a reduced egg hatch of the mated female but also as reduced survival of progeny during later developmental stages. In total about 50% of zygotes fail to develop to fertile adults. The sterility in the male is due to unbalanced gametes segregating from the translocation. In mosquitoes, males carrying these types of translocations have been used for control without the use of radiation (Laven, Cousserans & Guille, 1971). Steffens (1983) suggested that because of this inherent sterility, a reduced level of radiation could be used to sterilize the male. This would obviously have positive effects on the competitiveness of the sterile males in the field. Unfortunately, the dose response kinetics and chromosomal basis of dominant lethal mutations suggest that an additive effect of translocation and radiation induced sterility will not be found (Franz, 2000). Radiation induces dominant lethal mutations in normal sperm and in sperm carrying unbalanced chromosomes at equal frequency and in an exponential manner. This means that at increasing doses the same proportion of unbalanced and normal sperm carry a dominant lethal mutation and full sterility will only be reached when the chance that both types of sperm carry a dominant lethal mutation is 100%, that is, at the same dose. However, there is now increasing awareness that in many cases a lower dose of radiation should be considered for SIT (Rendon, Franz & Wood, 1996; Franz, 2000).

Advantages of using GSS in field programmes: testing assumptions

Reducing costs of rearing, emergence, handling, release and monitoring

For many insect species this economic consideration plays a major role in the consideration of developing a GSS for use in SIT. Under optimal conditions and assuming that female eggs could be killed by a simple and cheap procedure then savings up to 50% could be contemplated for the rearing, emergence, handling and release components of an SIT programme. For the emergence, handling and release components this saving is obviously realized as half the number of flies are

being handled and an aircraft will double its efficiency in releasing sterile males over a specified area. This saving is considerable as the costs associated with aerial release can be up to 15% of all programme costs. Potential savings in rearing costs are not so clear-cut. There will be some cost, however minor associated with the sexing procedure itself. For a pupal sexing system the costs of installation, maintenance and operation of optical sorting machines can be considerable. As illustrated above, the reduced fertility of males and the possible reduced fitness of mutant females will sometimes necessitate the use of a large adult colony.

Monitoring and evaluation of releases becomes greatly simplified when only males are released. In a medfly SIT programme flies for release are marked with fluorescent dye so that following sampling in the field the released flies can be differentiated from the field flies. This enables the progress of the programme to be monitored. Following a bisexual release both male and female marked flies are trapped and have to be differentiated from unmarked male and female wild flies. The differentiation of marked from unmarked flies is a very laborious procedure and subject to human error. The misclassification of a released fly as a wild fly can initiate a whole series of actions that are in fact not required. When mostly males are released it means that any female that is trapped is from the wild population and is therefore a direct measure of its size. The use of more specific female attractants (Epsky et al., 1999) greatly increases the usefulness of an all-male release. In theory, it should also be possible to compare the male:female ratio in the traps before release with that following an all-male release and use the difference to calculate the sterile:wild male ratio. This would remove the need for marking the flies as is now done in the medfly SIT programme in Madeira (Pereira et al., 2000). In medfly SIT programmes flies are generally held for some days to become sexually mature before release, this can lead to extensive mating of the males before release using a bisexual strain. When only males are produced, they are released as mature virgin flies.

Increased effectiveness in the field

The extensive series of open field studies carried out by Rendon et al. (2000) have shown unequivocally that an all-male release is 3–5 times more effective in inducing sterility in a field population than when both males and females are released. However, using the standard field cage evaluation system, the removal of sterile females from the released flies does not lead to greater proportion of females being mated (Cayol et al., 1999). This is little paradoxical but probably reflects the limitations of the system to mimic the overall behaviour of the fly in the spatial and temporal environment of an open field.

Field trials of sexing strains

An historical review of GSS in medfly can be found in Robinson, Franz and Fisher (1999) and only a summary is presented here. The first field tests in medfly were carried out on Procida Island, Italy in 1986 using a pupal colour GSS and both field cage and open field experiments were carried out (Cirio, Caparella & Economopoulos, 1986; Robinson et al., 1986). There were serious problems with the stability of the strain during mass rearing which severely limited the open field evaluation (Hooper, Robinson & Marchand, 1986). In Israel, which has an intensive citrus production system, aerial bait-spraying of insecticides is used to control medfly. Using a pupal colour GSS, the release of sterile males was compared with the use of conventional chemical control in a field experiment carried out in 1989–1990 in the northern Negev (Nitzan, Rossler & Economopoulos, 1993). The percentage of fruit with sterile stings and the percentage of fruit with live maggots were monitored for two years and the male releases compared very favourably with the insecticide treatment. The efficiency of these strains in an SIT programme for the control of medfly paved the way for the current wider application of this technique in Israel using a *tsl* GSS (Rossler, Ravins & Gomes, 2000). In large field experiments in Hawaii, McInnis et al. (1994, 1996) were able to demonstrate unequivocally that the release of predominantly males caused a 3–5 fold increase in the amount of sterility induced in the wild population compared to when males and females were released. The results of these series of experiments were instrumental in gaining a wider acceptance of the use of GSS in SIT programmes. The first field experiments using a *tsl* GSS were carried out in Greece (Hendrichs et al., 1993) and survival and dispersal were shown to be not different from observations on a bisexual strain. Hendrichs et al. (1996) carried out further studies of male competitive behaviour and they showed that males from all strains exhibited normal sexual

behaviour, attracted a similar number of female visits and had similar pheromone calling profiles throughout the observation period. Cayol and Zarai (1996) completed the initial field evaluations of medfly GSS in Tunisia.

These field experiments together with extensive testing in field cages using wild populations from different parts of the world has led to adoption of medfly GSS in almost all medfly SIT programmes.

Operational programmes using GSS

Guatemala

The El Pino facility in Guatemala has taken the lead in employing GSS strains for medfly SIT operational programmes. It is also the largest fly rearing facility in the world and currently produces 1 billion medfly males/week. The first GSS (VIENNA 4/Tol-94) was introduced in 1994 and it was based on the *tsl* mutation and the strain was backcrossed with Guatemalan flies before being mass reared. In 1997, the FRS was introduced to improve the management of the strain that was being reared to a level of 500 million males/week. In 1999, a new strain was introduced (VIENNA 7/Tol-99) employing another translocation which improved stability and the QC profile, it was also outcrossed with Guatemalan flies. This strain is still currently being reared and it is projected that 1.6 billion males/week will eventually be produced.

Argentina

The successful medfly SIT programme in Mendoza, Argentina (De Longo et al., 2000) has been using a GSS, SEIB 6-95, based on *white pupa* since 1995. Using pupal colour separators, up to 100 million males/week have been able to be released. The programme has also supplied sterile males to other programmes within Argentina. The programme changed to a *tsl* based system in 2001.

Madeira

The current medfly SIT programme on the island of Madeira is interesting as it is a suppression programme and is not aimed at eradication (Pereira et al., 2000). From its initiation in 1997, the programme has used a series of GSS. Initially, VIENNA 6/96 was used followed by VIENNA 7/Mix-99, VIENNA 7/Tol and from 2001 it has been rearing VIENNA 7/Mix-2000. All these GSS are based on the *tsl* mutation. The rather regular strain replacements have been necessary due to problems in this facility with maintaining strain integrity before the introduction of the FRS.

Australia

Medfly is established in Western Australia and in 1985 there was a successful pilot eradication programme for an isolated population of this pest in Carnarvon, 1000 km north of Perth (Fisher, Hill & Sproul, 1985). As part of a feasibility study a state-wide eradication, a new field evaluation of the technique, has been carried out in 1999–2000 in Broome (Woods, pers. comm.). In this programme the GSS VIENNA 7/Mix 99 was used. This strain carries genetic material from eight different geographic areas and up to 5 million males were released/week. Recently males from the facility in Western Australia are being used to help in the eradication of medfly outbreaks in South Australia.

South Africa

A feasibility study of using the SIT for medfly control in the Western Cape in South Africa is underway in the Hex River Valley. Initially flies were shipped from Guatemala but problems relating to a guaranteed supply, the quality of the shipped flies after long distance shipment and cost, encouraged the programme to develop its own rearing capacity. The programme is currently rearing VIENNA 7/Mix 99 with the aim to produce 6 million males/week. The programme so far has been very successful and it has a major impact on the export of agricultural products to the US (Annual Report ARC LNR 2000–2001).

Other operational activities related to the use of GSS

(a) As noted above, the medfly preventative release programme in California initially used both bisexual and all-male releases however, in the near future the majority of releases will be done using only males. This will be possible by the conversion of two large rearing facilities in Hawaii to GSS *tsl* rearing in 2001.

(b) The first large-scale medfly SIT programmes were carried out in Mexico in the early 1980s using flies produced from the Metapa rearing facility in Tapachula, Chiapas, Mexico (Hendrichs et al., 1983). The facility is still in production and is currently using a bisexual strain. In 2000 plans were drawn up to introduce a *tsl* based GSS which will

require remodelling of parts of the facility. The conversion of this facility to GSS rearing will mean that all major medfly SIT programmes use GSS.

(c) There are plans to develop a large medfly SIT control programme in the Middle East (Rossler, Ravins & Gomes, 2000) and a model project was initiated in 1997 in the Arava Valley. As there are no fly rearing facilities in the area for this programme, they are being shipped from Guatemala. A total of 662 million male pupae were shipped up until July 1999 and sterile males are being released over both the Jordanian and Israeli Arava valley. There are now recognised fly-free status for certain areas in Israel and vegetable products can be shipped to the US.

Future developments

The potential use of transformation to produce GSS is dealt with by Handler in this volume. It is planned that two major improvements will be introduced into GSS technology in the near future. Firstly, chromosomal inversions (Gourzi et al., 2000) will be used to add further stability to GSS by eliminating the gametes or zygotes which are produced following recombination in inversion heterozygotes (Robinson, 1975). Inversions will also facilitate the backcrossing of GSS to different genomes as translocation and the selectable markers become more tightly linked. Secondly, by using translocations in which the breakpoint on the Y chromosome is between the maleness factor and centromere, any surviving zygotes that originate from adjacent segregation will be female and mutant and thus will not compromise the quality profile of the male production.

Monitoring the progress of an SIT programme generally requires that released insects are marked with a fluorescent dye but this method is associated with some drawbacks (see Hagler & Jackson, 2001) mainly because it does not give absolute security. A genetic marker would solve this problem and the use of transformation to facilitate this is described by Handler (this volume). Another method to mark insects for release is to use a morphological mutation and evaluation of such a marker in combination with a GSS is underway (Niyazi et al., in prep.).

One observation following the use of *tsl* GSS in SIT programmes is that not every mass rearing location has the necessary expertise to develop the strains and to maintain their integrity over many generations of large-scale mass-rearing. Maintaining the integrity of a strain requires the faultless operation of the FRS to produce flies for the production colony. However, the mass rearing of males for release is fairly straightforward. The separation of these two processes could have certain advantages and can be achieved if eggs, from a facility implementing an FRS and holding a production colony, could be shipped to other facilities where they are heat treated and the males are reared, sterilized and released. The shipment of eggs for this purpose is currently undergoing evaluation (Caceres, pers. comm.).

As stated in the introduction, improvement of GSS in medfly SIT programmes will be a continuous process as will the development of these strains for other species. Based on the results of the work in medfly it should be possible that, given reasonable resources and a commitment to succeed, these types of strain can be developed for most insect species.

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