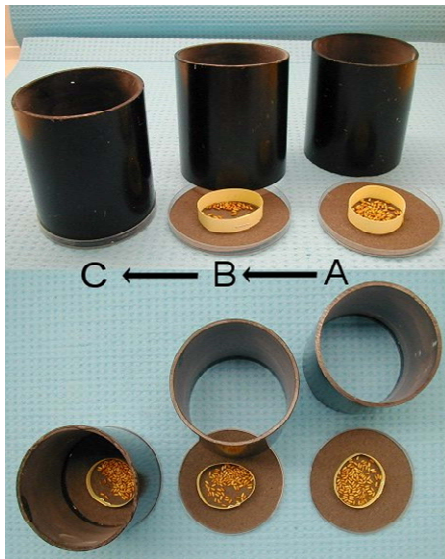# Product Quality Control for Sterile Mass-Reared and Released Tephritid Fruit Flies

Version 7.0

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IAEA, Vienna, Austria

Preamble to Version 7.0

Since 1997, the FAO/IAEA/USDA manual on "Product Quality Control and Shipping Procedures for Sterile Mass-Reared Tephritid Fruit Flies" has provided and continues to provide an objective set of standards for assessing quality of sterile fruit flies used in sterile insect technique (SIT) programmes. The manual addresses an essential need by those working in fruit fly control programmes to measure insect performance in concert with other operational activities. Use of insects of good quality equates to successful pest control. Conversely, use of poor quality insects can lead to a lack of effective control and higher programme costs. Measurement of insect quality in the field is the only way to provide the information to mass-rearing facilities so that they can modify or improve rearing protocols to maintain or improve insects quality.

The procedures set forth in this manual are based on published scientific findings for the most part. In the absence of peer-reviewed findings, expert opinion based on many years of experience at field SIT action programmes and consensus reached through open dialogue also played a role determining the recommended practices cited. This is particularly important where transboundary shipment and release of sterile insects occurs. It also bolsters confidence in the production and use of sterile insects especially where private sector investment in mass-rearing facilities and fly production and release is involved.

This manual is a living document and is subject to periodical updates; the most recent version is available on the Internet at http://www-naweb.iaea.org/nafa/ipc/public/manuals-ipc.html. The following events and activities have led to the current revision of the manual:

- 2000-2004 FAO/IAEA Coordinated Research Project: Quality Assurance of Mass Produced and Released Fruit Flies

- 2003-2009 FAO/IAEA Coordinated Research Project: Development of Mass Rearing for New World (*Anastrepha*) and Asian (*Bactrocera*) Fruit Flies.

- 2003-2009 FAO/IAEA Coordinated Research Project: Improving Sterile Male Performance in Fruit Fly SIT programmes.

- 2004, 2006, 2008 and 2012. Meetings of the Working Group on Fruit Flies of the Western Hemisphere.

- 2005 "Sterile Insect Technique. Principles and Practice in Area-Wide Integrated Pest Management" is published. V.A. Dyck, J. Hendrichs and A.S. Robinson (eds.). 787 pp.

- 2006, 2010, 2014 and 2018 . 7th, 8th, 9th and 10th International Symposia on Fruit Flies of Economic Importance.

- 2007 "Area-Wide Control of Insect Pests: From Research to Field Implementation" is published. M.J.B. Vreysen, A.S. Robinson and J. Hendrichs (eds.) 789 pp.

- 2007 FAO/IAEA Coordinated Research Project: Quality Assurance of Mass Produced and Released Fruit Flies (Proceedings published as a special issue in the Florida Entomologist).

- 2007 FAO/IAEA "Guidance for packing, shipping, holding and release of sterile flies in area-wide fruit fly control programmes" is published. W Enkerlin (ed.). 134 pp.

- 2008 and 2013. Tephritid Workers of Europe Africa and the Middle East meeting with proceedings in a special issue in the Journal of Applied Entomology.

- 2009. USDA-APHIS. United States, Mexico and Guatemala Fruit Fly Emergence & Release Facilities Review - Final Report. Conducted July 2008. 81 pp.

- 2012. Santiago Martínez, G. et al. Manual de Control de Calidad de moscas esteriles: Procedimientos para evaluar el producto en centros de empaque. Dirección de Moscas de la Fruta, DGSV-SENASICA, SAGARPA 34 pp.

- 2013. "The FAO/IAEA Spreadsheet for Designing and Operation of Insect Mass Rearing Facilities", C. Cáceres, P. Rendón and A. Jessup (ed.). FAO, Rome, Italy, 48 pp.

- 2013. Jorge C. Guillen Aguilar et al. "Manual to Differentiate Wild, Fertile Mediterranean fruit flies, *Ceratitis capitata*, from Irradiated, Sterile TSL flies", FAO/IAEA, Vienna, Austria, 55 pp.

- 2016 FAO/IAEA. "Guidelines for the Use of Mathematics in Operational Area-Wide Integrated Pest Management Programmes Using the Sterile Insect Technique with a Special Focus on Tephritid Fruit Flies". Barclay H.L., Enkerlin W.R., Manoukis, N.C. Reyes-Flores, J. (eds.), Food and Agriculture Organization of the United Nations. Rome, Italy. 95 pp.

- 2018. FAO/OIEA. Manual para diferenciar moscas de Anastrepha ludens (Loew) silvestres y criadas de cepa normal ("bi-sexual") y cepa sexada genéticamente (Tapachula-7), irradiadas y sin irradiar, by Guillen Aguilar J.C., Lopez Muñoz L., Lopez Villalobos E.F. y Soto Garcia D. N. Viena, 95 pp.

- 2019. FAO/IAEA. Sterile Insect Release Density Calculations Spreadsheet, Rendón P.A, Enkerlin W.R. and Cáceres C. (eds.), Food and Agriculture Organization of the United Nations/International Atomic Energy Agency. Vienna, Austria. 30 pp.

Table of Contents

1. Introduction

1.1. Scope

This document represents the recommendations, reached by consensus of an international group of quality control experts, on the standard procedures for product quality control (QC) for sterile mass-reared and released tephritid flies that are to be used in Sterile Insect Technique (SIT) programmes, both at the mass-rearing facilities and fly emergence and release facilities. This international manual describes recommended procedures for sampling, irradiation, dosimetry, packaging and shipping. A flowchart showing the sequence of routine and periodic QC test to be carried out at the mass-rearing and fly emergence and release facilities is presented (**Figure 1.1**).

A majority of the procedures were initially designed specifically for the Mediterranean fruit fly, *Ceratitis capitata* (Wied.), but they have been included now for several other species, such as *Anastrepha* and *Bactrocera* species, and are applicable, with minor modification, to other tephritid species.

If followed, tests and procedures described in this manual will help ensure that the quality of mass-produced flies is measured accurately in a standardized fashion, allowing comparisons of quality over time and across rearing facilities and field programmes. Problems in rearing, irradiation, packing, shipping, handling and releasing procedures, and strain quality can be often identified by closely monitoring QC results thus allowing for corrections to be made before field programmes are adversely affected. Although routine and periodic QC testing can reveal measurable changes (both positive and negative), it is less definitive in pinpointing the causes of reduced performance or quality. For example, insect weight, flight ability, and mating propensity can be affected by one or multiple causes. Nevertheless, any sudden decrease or observable changes over time that result in lower than expected results should stimulate a search to identify possible causes. QC tests also play a role in monitoring and measuring recovery to normal levels.

Tests and procedures described in this document are only part of a total quality control programme for tephritid fly production and release that can also be used as a validation for quality assurance for rearing and release processes. A list of tephritid fly rearing facilities worldwide is available at: http://nucleus.iaea.org/dirsit/ in which each facility is encouraged to upload their current operational procedures. The *product* QC evaluations included in this manual are intended to be carried out at both the mass-rearing and the fly emergence and release facilities. Both parties benefit in comparing and assessing results of routine and periodic QC tests to see: (1) what positive effects to sterile fly performance may result in making production changes or improvements (new strain assessments; nutritional, microbiological, semiochemical and hormonal supplements; etc) and (2) what quality loss may have occurred during packing, shipping, holding and release.

REQUIRED PRODUCT QUALITY CONTROL TESTS

	Daily/weekly ("routine")	Monthly /annually ("periodic"))

MASS REARING FACILITY

Mass-Rearing — Sampling procedures

- Pupal weight
- Emergence and flight ability
- Survival under stress

Irradiation — Irradiation procedures

- Radiation indicator

  - Sterility

- Emergence and flight ability
- Survival under stress
- Sex ratio and timing of emergence

  - Mating performance in field cage
  - Fly irritability
  - Cage Fried

Shipping

FLY EMERGENCE AND RELEASE FACILITY

Arrival — Sampling procedures

- Radiation indicator

  - Sterility

- Pupal weight
- Emergence and flight ability
- Survival under stress
- Sex ratio and timing of emergence

  - Mating performance in field cage
  - Fly irritability
  - Release-recapture tests of dispersal and survival
  - Cage Fried

Handling/Chilling

- Adult fliers
- Survival chilled/released flies under stress

Release

  - Adult fliers
  - Post-release Survival stress

Figure 1.1: Flowchart of the product quality control for sterile mass-reared and released tephritid flies.

Additional product QC tests have been developed and their use is optional (see Chapter 8 Ancillary tests). These tests have more of a research character and should be carried out mainly to understand the causes of specific problems. *Production* and *process* QC evaluations (e.g. analysis of diet components, monitoring the rearing environment, yield of larvae, development rate, etc.) are not within the scope of this document. Standard Operating Procedures (SOPs) used by mass-rearing facilities to conduct *production* and *process* QC evaluations are available online at: http://nucleus.iaea.org/ididas/

The manual continues to evolve and is subject to periodic updates. Future additions will include new tests, other fruit fly species and/or stages at which tests should be run as the need is identified and data become available. Previous versions of the manual contained minimum and mean acceptability specifications of conventional and genetic sexing strains for the main SIT target species of Tephritidae (e.g. *C. capitata* and some *Bactrocera* and *Anastrepha* species). Further updates of acceptability levels and incorporation of standards from new species rely on the information provided by the rearing and fly emergence and release facilities. This expansion is being driven by the need for more environmentally compatible pest control methods, lower costs in the production of sterile insects, and increased availability of sterile insects stemming from a number of newly constructed facilities with greater production capacity. Private sector investment has advanced commercial production and sale. This is based on greater confidence in the technology, but more importantly on the quality of the product. It also important to mention that there is a greater need today to increase production of healthier, nutritious foodstuffs free of pesticide residues to meet an ever-increasing global demand. Apart from demonstrated effectiveness of SIT to control pests, there also are important environmental benefits derived from reducing dependence on use of insecticides.

1.2. Background

It became evident in several SIT fruit fly projects in the 1960s and 1970s that mass-reared fruit flies were not performing in the field as expected. In an effort to define and measure the performance of mass-reared flies and wild flies, tests were developed by various workers (Chambers 1975). Scientists gave particular attention to the effects of gamma radiation on the insect and its ability to mate successfully. In some instances, non-irradiated mass-reared insects also performed poorly shifting attention to seek other causes for poor performance especially in the production processes. For almost 35 years (see Appendix A: Chronology of Product Quality Control of Tephritid Flies for Use in SIT Programmes), systematic efforts by many groups and individual scientists successfully developed a series of simple and useful tests to compare the quality of sterile tephritid flies produced at different rearing facilities. Those efforts led to the production of several fruit fly quality control manuals (e.g. Orozco *et al.* 1983, Boller and Chamber 1984, Brazzel *et al.* 1986). With increased production and use of sterile insects coupled with recent investments, public and private, in construction of rearing and release facilities, there is an on-going need for universally accepted standards of quality against which sterile insects can be measured objectively. QC testing can also provide evidence of programme cost effectiveness, an important consideration from a budgetary or investment standpoint. As done previously to address these needs, the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture convened a Consultant's Meeting in October 2010 and later in December 2015 to review and revise this manual.

1.3. Rationale

1.3.1. The Need for Product Quality Control in SIT Programmes

The goal of SIT programmes is to reduce or eliminate wild insect populations. These programmes are effective when high proportions of wild females mate with sterile males and thus fail to reproduce. Successful application of the SIT requires that an effective ratio of sterile to wild flies (the "over flooding ratio") be maintained in the field.

Managers of SIT programmes also need to ensure that, once in the field, the sterile males compete effectively with wild males and mate with wild females and successfully transfer their sperm. This is especially critical for insects that, like tephritids, in general have a complex mating system. Effective methods for monitoring and providing timely feedback on the quality and competitiveness of sterile fruit flies are critical to the success of SIT programmes. Doing so could result in significant gains both in efficiency and effectiveness. Conversely, disregard for insect quality could lead to substantial programme cost increases and a lack of effective pest control.

1.3.2. Mating Behaviour and Sterile Male Competitiveness in the Field

Competitiveness is the overall ability of sterile males to compete for wild females against wild males of the target population. The components of competitiveness can be lumped into broad categories such as ability to survive in the field, mating propensity, mating compatibility, and post-mating factors. The complexity of these various categories, and their relative influence on sterile insect competitiveness, will vary depending on the biology and mating system of the species. A comprehensive QC programme for the mass-reared males (end product) should contain a full scope of tests designed to establish fly competitiveness. Tests that are used must be tailored to, and appropriate for, the individual species. To that end, QC tests should ideally be capable of measuring quality parameters at each stage of the process starting with mass-rearing, sterilizing, shipping, arrival at the fly emergence and release facility, handling/chilling and release as noted in the flowchart (**Figure 1.1**).

Mating compatibility is defined as the successful interaction of the sterile male and wild female fly, from behaviours that bring them together in a mating arena, resulting in effective sperm and accessory gland products transfer. In SIT programmes for pest tephritids, mating compatibility is essential because of two principle characteristics of their mating systems. First, the behaviour performed by the male in these mating systems is complex; second, females actively choose a mate from a number of suitors. The following discussion of mating behaviour is specific to *C. capitata* but relates, in principle, to many other pest tephritids that are subject to SIT operations.

In order to be accepted by a wild female to mate, a sterile male must initially locate a microhabitat suitable for a mating arena and then begin "calling" (releasing sex attractant pheromone). For Mediterranean fruit fly, the microhabitat is typically on the underside of a well-lit leaf, and locating it probably involves responses to light and other physical stimuli. The multi-component pheromone presumably plays a role in courtship (see below) as well as in luring unmated females. Males usually call in small aggregations that Prokopy and Hendrichs (1979) referred to as leks. Since then, most fruit fly workers have applied the term lek to the mating system of *C. capitata* and other tropical Tephritidae.

When a female approach, the male begins his courtship which may involve chemical (pheromonal), visual, acoustic, and tactile cues. Qualitatively, the ritual is very stereotyped (the same subset of behaviours almost always occurs), but quantitatively, there is plenty of variation to allow for distinct differences among males, both within and between populations. If females show interest in the male during the courtship or simply remain in the vicinity long enough, the male will usually attempt to mount her. Even if the male mounts the female, she still may choose to shake him off rather than mate. A female typically solicits courtships from a number of males before allowing a male to mate with her.

Laboratory colonization and mass-rearing can result in changes in the behaviour of mass-reared males. The changes potentially influence such factors as mating age, courtship behaviour, quality or quantity of pheromone produced, and diel periodicity (Cayol 2000). If wild females fail to come in contact with, or reject, sterile males because of these changes, the competitiveness of the sterile flies can be drastically reduced, as shown for a laboratory

colony of more than 40 years (McInnis *et al.* 1996). Some of these changes have genetic bases; i.e., result from inadvertent selection or genetic drift in the rearing colony.

1.3.3. Changes from the Earlier Manuals

As shown in the Product Quality Control flowchart (**Figure 1.1**), the new manual has been reorganized into two parts: (a) mass-rearing facilities and (b) fly emergence and release facilities. Required routine and periodic tests are identified for each of the major processes by facility. Absolute fliers post handling/chilling and post release at fly emergence and release facilities were added as required routine tests. Mating performance in field cages and capacity to evade predators were added as a periodic test at mass-rearing facilities. The following required routine tests were renamed: pupal weight (in place of pupal size); survival under stress (in place of longevity under stress). Ancillary tests have been moved from the Appendix into the main body of the manual as a separate section (section 8). The following tests have been added as new ancillary tests: activity test, effect of chilling on absolute fliers, male calling time, and incidence of remating. Laboratory mating test has been removed from the manual.

Previous manuals (Orozco *et al.* 1983, Brazzel *et al.* 1986) were intended to provide standardized procedures for routine (daily, weekly) checks of the product of the rearing process. The tests that they outlined were, accordingly, designed to assess emergence, flight ability, mating propensity and indices of the basic viability of the mass-reared flies. In particular, tests outlined by Brazzel *et al.* (1986) were not intended to address mating competitiveness and compatibility or post-mating factors, although the authors noted the need to run regular tests in those areas.

In the years that followed, results of the tests outlined by Brazzel *et al.* (1986) apparently became equated with overall fly quality and competitiveness, at least for Mediterranean fruit fly SIT programmes in the United States. Later research (e.g. Hibino and Iwahashi 1989, 1991, Miyatake and Yamagushi 1993, McInnis *et al.* 1996) suggested that a decline in mating compatibility was survive long enough in the field and a possible cause of poor performance by sterile males in the field and, when severe, can result in the failure of SIT programmes.

In 1997, FAO/IAEA, USDA and other experts consolidated quality control tests into a common set of standards for use internationally rather than for specific programmes. Distinctions were made between required routine and periodic tests, as well as those considered to be ancillary in nature. The aim was to identify a few essential robust tests that also could be carried out in a simplified fashion with materials accessible in most countries at a relatively low cost. A standard format was adopted for all tests: descriptive name, objective, discussion, equipment and supplies (where applicable), procedures and interpretation. Each test was given a stand-alone format providing flexibility to easily reclassify, move or remove existing tests while making it easy to insert new tests into the manual as appropriate.

The impetus to revise the current manual stems from the need to update performance specifications for many of the species and to add new tests, mainly for fly emergence and release facilities, that will aid fruit fly workers to better assess insect product quality and make better informed decisions to improve SIT programmes. A number of advances have occurred particularly in the handling, packaging and shipping that can improve the manner in

which product quality at each step in the mass-rearing, irradiation, shipping, arrival handling/chilling and release is more accurately measured. Irradiation and dosimetry procedures for the X ray sterilization have been added for the first time.

1.3.4. Management of Product Quality Control

Conflicts may arise in the production facility with the need to mass-rear predetermined quantities and at the same time to produce high quality flies. Examples include: (1) attempts to increase production levels may result in reducing the size and quality of the sterile flies, (2) production managers may not find it advantageous to report or admit to lapses in the quality, and (3) production managers might be hesitant to replace an older strain with a newer less laboratory-adapted strain that is initially more difficult to rear but more competitive in the field. Because of these conflicts, it is strongly recommended that *product* QC for SIT programmes be conducted by a unit that is not directly responsible for the production of the sterile flies (Calkins at al. 1996). The *product* QC unit should not report to the Rearing Manager but to the Programme Manager. However, the *product* QC unit must work closely with rearing personnel involved in *production* and *process* QC evaluations and provide continuous feedback necessary to maintain an effective rearing process. Post-irradiation QC evaluations that are conducted at the production facility should be corroborated at emergence facilities and at field release sites utilizing the same methodology.

The most important part of the QC programme is to ensure that the mass-reared sterile males interact successfully with the wild females of the target population. To ensure that the sterile males are competitive and compatible with the wild females, field evaluations must be conducted routinely. These tests should include wild flies collected from the area where releases are to occur or conducted in the location that is a likely source of pest introductions. Because this activity is critical to programme success, sufficient funding and other resources must be allotted for this purpose. The full-time staff dedicated to conducting field evaluations should include personnel trained in behavioural and ecological aspects of fruit fly biology. Both the Programme Manager and the end user (increasingly not the same) should be responsible for ensuring that these tests are conducted, reports are submitted to all concerned parties, and appropriate actions are taken.

1.3.5. Future Plans to Update this Manual

Since 1997, several revisions to the manual have occurred and will continue into the future as improvements are made in production, shipping and use of sterile insects. In this regard, ongoing efforts by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, especially by sponsoring Coordinated Research Projects on an international level, will continue to play a key role in the development, implementation and improvement of the sterile insect technique on a global level.

This international manual and future revision will become available through the Joint FAO/IAEA Division Home Page on the Internet (http://www-naweb.iaea.org/nafa/ipc/public /ipc-mass-reared-tephritid.html).

1.4. Data Analysis, Presentation and Communication

Simple, routine procedures are available for collecting and preparing data on product quality that allow producer and user to evaluate the continuing processes of production and to predict trends leading to reduced quality. It is recommended that greater attention on equipment calibration will increase reliability in data collection. In addition, more attention should be placed on in-depth analysis of the data to determine where methodologies can be simplified and frequency of testing can be adjusted.

To achieve this, mass-rearing and fly emergence and release facilities should:

Prepare a capability analysis of each of the variables identified in the list of required routine quality tests. A sample of ≈50 independent (separate batches) measurements of each parameter are needed during a period when the rearing process is thought to be "under control" (i.e., when fly quality is, subjectively, "good") to establish a reference standard. Mean values or overall proportions from each of those 50 test results are then used to compute a measure of central tendency (for example, overall mean) and control limits. Typical lower control limits might include a "warning limit" (set at a level where, with normal variation, measurements would be expected to exceed the warning limit 97.5% of the time) and an "action limit" (99.5% level). For continuous variables (e.g. pupal weight), the "warning" and "action" limits would, for example, be 2 and 3 standard deviations below the mean. For data in which each individual falls into one of two categories (for example, emerged or unemerged, male or female), statistical procedures based on the binomial distribution (for example, *P*-charts) may be more appropriate.

Routinely (e.g. weekly) produce graphs that track the values of each QC parameter over time. Graphs should also show lower control limits and perhaps the minimum levels specified in the respective section of this manual.

Provide copies of the charts to users and rearing personnel. Values below the action limit, or consecutive values below the warning limit, would indicate a problem with the production and post-production processes that requires the attention of rearing personnel. Extended periods when measurements consistently fall below the central value also indicate potential problems. Charts are provided to the user to enhance communication regarding variations and trends in fly quality. Analysis and charting of each measured parameter at the receiving station will be similarly conducted to allow comparison.

With advances in computer technology, entry, storage, manipulation, and graphing of QC data can be easily automated. Data can be stored conveniently in electronic databases (use secure back-up systems), and database, dashboard, or spreadsheet forms can be designed that mimic the QC Forms such as those presented in this manual. Statistics such as flight ability, percent emergence, and mating propensity can be computed automatically, reducing the chance of human error. Data can be exported to spreadsheets or other applications for routine analysis or production of graphs. A number of statistical software packages are available that include specific routines for computing control limits and producing quality control graphs.

Control limits are effective for identifying deviations from normal levels in production processes because they are based on statistical evaluations of production data from a given facility, process, and strain of fly.

1.5. General Comments

A decrease in the efficiency of the SIT can originate from different aspects related to the quality of the males: capacity to disperse, survive, forage for food and nutrients that have to be incorporated as adults and, of course, court and mate with wild females. When reduction in any of these aspects is detected at the rearing facility some corrective actions can be undertaken. Increasing the number of insects released is one option when the survival rate and dispersal ability are compromised. However, if the reduced quality of the insects is related to their ability to court and mate with wild females any increase in the release ratio will not compensate for this deficiency and other corrective actions should be considered. The set of tests provided under the Required Periodic and Ancillary Tests provide the first insight on overall competitiveness of mass-reared sterile males. Although not performed in every batch of flies, they should not be overlooked, and information recovered in each of them should be analysed in conjunction with the results from the others. It is highly recommended to perform Ancillary Tests along with the Required Periodic Tests in order to have a more complete panorama of the quality of the flies. However, knowing the amount of work these tests require, it is advisable also to compare data from different years and if applicable different fly emergence and release facilities which receive flies from the same rearing facility in order to adjust the frequency for performing the ancillary tests.

1.6. Acknowledgements

A revised version of Brazzel et al. (1986) was used as a basis for the development of this document. Agencies involved in the initial harmonization included FAO/IAEA (Austria), Moscamed-Guatemala (Guatemala), Moscamed-Mexico (Mexico), USDA (APHIS-IS, APHIS-PPQ, ARS), CDFA (California), and FDACS-DPI (Florida).

Procedures and specifications described herein are taken from the cited literature and/or are based upon work done at *Ceratitis capitata, Anastrepha, Bactrocera* and *Zeugodacus* mass-rearing, fly emergence and release facilities and/or research laboratories in Argentina, Australia, Austria, Chile, Croatia, Greece, Guatemala, Israel, Japan, Jordan, Mauritius, Mexico, Morocco, Peru, Philippines, Portugal, Spain and the United States (California, Florida, Hawaii, Texas).

Upgrades for Version 6.0 of this manual are based on a Consultants Meeting held in Vienna during October 2010 with fruit fly quality control experts from FAO/IAEA, Argentina, Australia, Guatemala, Mexico and the USA. Version 7.0 resulted from a revision of version 6.0 through a Consultants Meeting held in Vienna in December 2015 with fruit fly experts from Argentina, Australia, Mexico and the USA

1.7. Relevant Literature

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2. Required Routine Quality Control Tests at Mass-Rearing Facilities

2.1. Guidelines for Sampling Insects for Routine QC Tests

Objective

To obtain a sample of pupae or adults for product QC testing that is unbiased and representative of the insects that will be released in SIT programmes.

Discussion

Under optimal rearing conditions the quality of insects produced by a facility should not differ significantly from batch to batch even if variation occurs in factors such as nutritional or physical characteristics of the diet, microbial load, microenvironment proximal to the rearing unit, and even the technique of different personnel. Groups of insects that complete larval development more rapidly tend to be larger and of higher quality than those that develop more slowly. Therefore, a stable rearing system will result in consistent development time for all life stages.

Sampling Strategy

For *process* QC, undertaken by the production personnel in the rearing facility, the variation between rearing groups or units is important because the data from the testing serve to highlight and track rearing or handling problems which can then be avoided in future batches. For this to be possible, samples are taken systematically at random from each production batch and processed individually.

The requirements for *product* QC are different. The data obtained from product QC tests must give an accurate indication of the quality of the flies delivered to the field so that overflooding ratios can be maintained. As several batches or groups of insects are used to make up a shipment on any one day, it is imperative that the samples taken for product QC testing are representative of the *entire* shipment. To avoid biased and inaccurate test results, daily random samples are taken from across the pupae destined for irradiation and shipment, regardless of production batches or units. These samples should be pooled to reduce the sample-to-sample variability and, consequently, the number of samples required to achieve the desired level of precision in estimates of the means. *Product* QC is undertaken by the QC personnel at the mass-rearing facility before and after irradiation, and also by staff at the fly emergence and release facility.

Procedure

To be useful, the means of the parameters tested need to be estimated with some precision. There are numerous equations available to estimate the number of samples required to achieve the desired level of precision. All require that a mean, standard deviation and standard error of the mean be calculated from the samples (or obtained from recent data).

The precision of a mean can be estimated and the number of samples needed to achieve a desired level of precision can be calculated by the formula:

$$n_E = \left(\frac{s}{E\bar{x}} \right)^2$$

where n_E is the number of samples required

s is the standard deviation of the means

E is the level of precision required (or the standard error expressed as a decimal proportion of the mean).

For example, 20 samples of 100 pupae are weighed to give 20 estimates of mean pupal weight for a day's shipment. The mean and standard deviation of those 20 samples are computed and these values used in the formula above. Assume the mean and standard deviation to be 8 ± 1.5 mg.

To be 95% confident that the estimate of mean pupal weight is accurate to within ± 0.1 mg, the standard error would be ≈0.1/1.96 (where 1.96 relates to the 95% confidence interval about the mean), or 0.05 mg. E would equal 0.05/8, or ≈0.006, and the number of samples required would then be estimated at $(0.15/(0.006*8))^2 = 9.77$ (that is, 10 samples).

If this procedure were repeated several times on different days with similar results, a protocol for sampling pupae for determining pupal weight could be set at weighing 10 samples of 100 pupae each. The above formula for n_E will suffice if random or pooled samples (as described above) are taken. However, if stratified sampling schemes are used, more complex formulae are required for estimating the required sample size. This approach is not recommended.

There is a tendency to take many more samples than are really necessary in an effort to increase the precision of the estimate of the mean. However, from the relationship SE = s/√n, it is seen that as the standard error, SE, is inversely proportional to the *square root* of the sample size, n, it follows that a very large number of samples needs to be taken for even a small improvement in precision, and the time and cost involved are rarely justified in a mass-rearing facility.

In fact, evidence suggests that increasing the *number of pupae or individuals* in a sample may be more effective in reducing the variability between samples than increasing the *number* of samples taken, as found for *Anastrepha obliqua* and *A. ludens* at the Mexican facilities.

It is more effective to try to reduce variability between samples by ensuring that facility Standard Operating Procedures for rearing are maintained, and an appropriate sampling plan developed and adhered to. Consistent insect quality will ultimately produce more uniform estimates from fewer samples. Based on the above, it is recommended that individual facilities determine their own sampling size which give acceptable precision for the least number of samples to be processed, based on their own data.

2.2. Quality Control Tests after Mass-Rearing (pre-irradiation)

2.2.1. Pupal Weight

Objective

To obtain an accurate estimate of the mean weight of a group of pupae by measuring the weight of a sample.

Discussion

Weight is one of the first indicators of the stability and consistency of the mass rearing system; it is a valuable indicator of overall viability of pupae and correlates with size of the resulting adult flies. Evidence shows that larger mass-reared sterile male tephritids will, in general, be stronger fliers, live longer, have higher mating propensity and produce longer refractory periods in female flies than smaller males. Measured values of mean pupal weight will vary depending upon the strain and rearing system, so using weight to compare overall quality of pupae from different facilities must be done with caution. This evaluation should be conducted at the mass-rearing facility, and should be repeated on arrival at the fly emergence and release facility for confirmation.

Due to its practicability, most facilities have adopted pupal weight as the measure of size for product quality control. It is recommended that assessment of pupal weight be the required routine test for product size. , and pupal diameter be an ancillary test where appropriate. The procedure for assessing pupal diameter is described in Section 8.10 Pupal Size.

Test Frequency: Pupal weight, is easily and quickly assessed, and gives a robust measure of pupal quality. It correlates well with other quality parameters and can be a predictor for tests performed later. Because of this, it should be performed routinely for each irradiation batch or shipment.

Equipment

- Balance or scale with accuracy of ±1 mg or better (**Figure 2.1a**).
- Soft forceps for handling pupae and removing trash from samples.
- Board with ridges or grooves, or other device for simplifying the process of counting pupae (optional).
- Manual or optical seed counter (optional). An optical counter (**Figure 2.2b**) may be used for counting pupae in this test, but it must be calibrated to ensure accuracy.

Test Conditions

- Temperature 25 ± 1 ºC

- Humidity $65 \pm 15\%$ RH
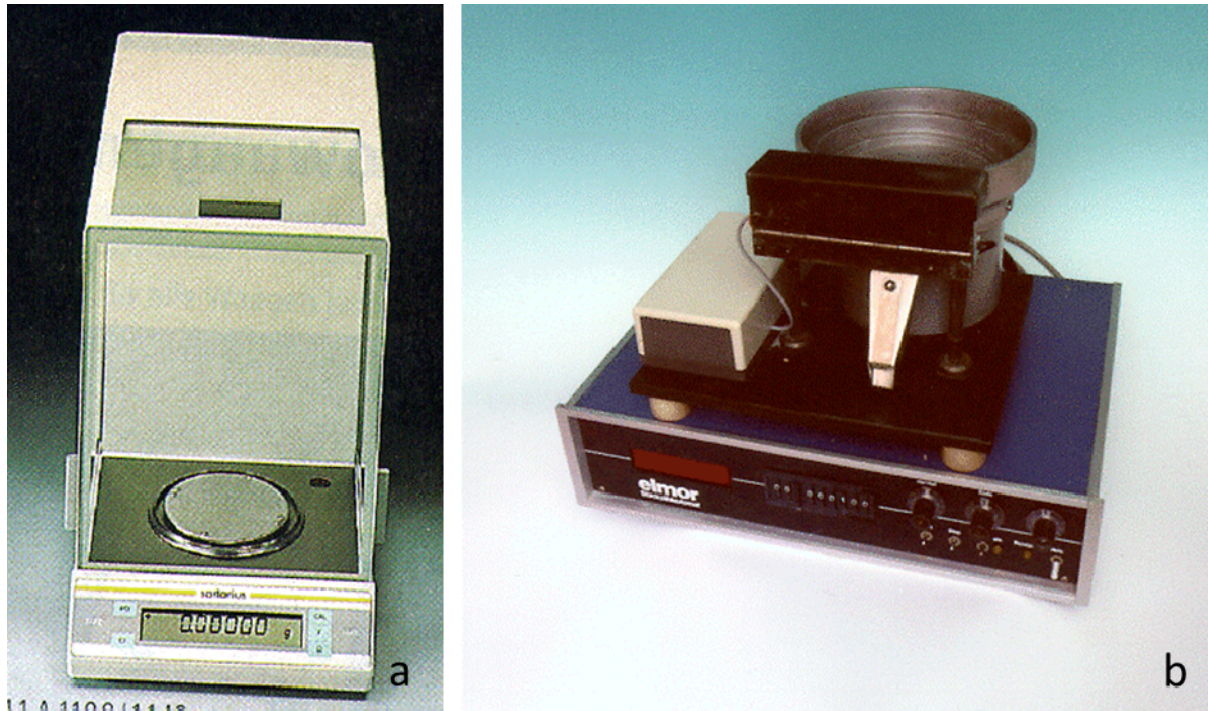
Figure 2.1: (a) A typical balance used to weigh pupae; (b) Automated pupal counter to count pupae in samples.

Procedure

Mean pupal weight is determined by taking samples few hours before irradiation and weighing several lots of 100 pupae (as in the case of *B. tryoni*). Alternatively, volumetric samples of pupae (e.g. 2 ml for *C. capitata* or 7 ml for *A. ludens* and *A. obliqua*) can be weighed and then counted. Age at sampling is critical because pupae lose water (and, therefore, weight) as they age; hence the requirement to perform this test at a set age each time. The number of lots that need to be weighed will depend on the desired level of precision in the measurement and the amount of lot-to-lot variation in weight (see Section 2.1 on sampling). Standard errors for estimates of mean weight of individual pupae should be ≈0.05 mg (95% C.I. of ≈0.1 mg) to ensure accurate estimates of numbers of flies released. Lot-to-lot variation will depend on the larval harvest method, the consistency of the rearing operation, and the method of sampling pupae for this test.

Note: care must be taken in counting the pupae (suggestion: count each lot twice and make sure the counts agree); a miscount of ± 1 pupa will produce an error of almost ± 0.1 mg in mean pupal weight for that lot.

A sample form for recording pupal weights is provided in 9.1 (Pupal Weight Assessment Form). The specifications for mean pupal weight of fruit flies produced for SIT programmes are listed in **Table 2.1**.

Table 2.1: Specifications for mean pupal weight of tephritid flies produced for SIT programmes.

Species	Pupal Weight (mg)	
	Minimum	Acceptable mean
Ceratitis capitata		
genetic sexing strain (tsl)	7.00	7.80
Anastrepha ludens	17.08	17.71
Anastrepha obliqua	13.52	14.22
Anastrepha suspensa	10.00	14.00
Zeugodacus cucurbitae	13.00	13.50
Bactrocera dorsalis	12.30	12.90
Bactrocera oleae	6.00	6.54
Bactrocera tryoni	8.50	10.00

Interpretation

Downward trends in mean weight of pupae produced by a facility can result from poor nutrition, overcrowding in the larval stage, high temperatures in the larval diet, larval diet microbial contamination, inappropriate holding conditions during pupal maturation (e.g. desiccation), or other factors that could reduce the viability of the released insects. As small size below a critical threshold (minimum pupal weight) is likely to be accompanied by poor performance on other quality indices and in the field it must be avoided by using specific rearing standard operating procedures for each species.

2.2.2. Emergence and Flight Ability

Objective

To obtain an accurate estimate of the percentage of adults that will emerge and percentage of adults that are able to fly.

Discussion

Sterile flies must be able to disperse from the point of release to locate shelter, food, water and, most importantly, mates. If they are unable to fly will therefore be useless to the SIT programme.

An accurate estimate of the number of fliers in a batch of sterile pupae delivered to a fly emergence and release facility assists in planning releases to achieve a desired overflooding ratio. The test is performed routinely on pupae before and after irradiation, and again after shipment for programmes with remote fly emergence and release facilities.

The test involves placing pupae in a tube and calculating the number of fliers from what is left in the tube at a set time after the adults have emerged. Examination of what remains in the tubes gives information on how many flies have emerged, have only partially emerged, are

deformed or are fully emerged but are non-fliers. This information is useful to evaluate and correct problems in rearing, age of irradiation, handling and shipping.

The test has been refined several times since it was first designed and now requires the flies to escape from the tubes unaided, rather than being lured.

A different procedure is used to assess the flight ability of sterile flies after chilling (see section 6.3.1) and after release (see section 6.4.1). For those stages the test is called absolute fliers since uses adults as a starting point.

Equipment

- Plexiglas tubes: outside diameter 8.9 cm with 3 mm thick walls; painted black (or of opaque black Plexiglas) so that light enters only at the top; 10 cm high. (**Figure 2.2**). Plexiglas has been chosen over cardboard or glass because it is unbreakable and can be washed and reused indefinitely. Care must be taken to prevent scratching the interior of the tubes whilst cleaning them. Avoid using an abrasive cleaner.
- Petri dish lids, 90-100 mm in diameter, should be painted black or the bottom overlaid with black paper (such as black filter paper).
- Strip of porous paper (such as construction or blotter paper), 1 cm wide, and formed into a ring 6 cm in diameter.
- Miscellaneous equipment: unscented talcum powder, hand counter, soft forceps, and micro-spoon spatula.
- Room/chamber with a controlled environment.
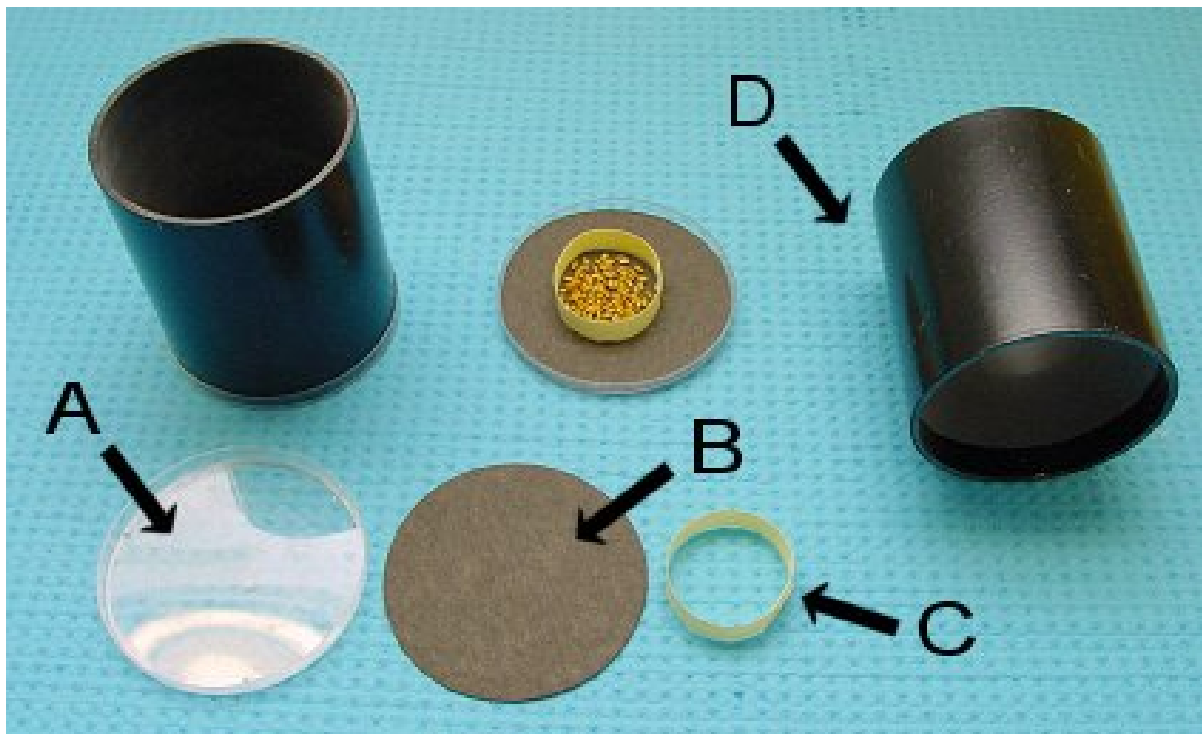
Figure 2.2: Equipment for the emergence and flight ability test.

Test Conditions

- Temperature $25 \pm 1°$ C
- Humidity $65 \pm 15\%$ RH
- Light intensity 1,500 lux (top of tubes)
- Photoperiod   14 hours light:10 hours dark

Test Frequency: Emergence and flight ability are assessed for every irradiation batch or shipment.

Procedure

Two days before emergence, 100 pupae are placed within the ring of paper, which is centred in the bottom of the Petri dish. Before each use, the inside of the tube is lightly coated with unscented talcum powder to prevent the flies walking out. Tubes are tapped on a firm surface to remove excess talc, and the talc should be wiped off of the bottom 1 cm (*Ceratitis* and *Bactrocera*) or 3 cm (*Anastrepha*) of the inside of the tube to provide resting places for newly emerged flies to set their wings. The wiping should be done with a gloved finger or a cloth to avoid introducing moisture or oil to the tube. The Plexiglas tube with talc is placed in the darkened Petri dish lid. Five replicates (five tubes with 100 pupae each) are set up for each lot to be tested. The test set up is shown in **Figure 2.3**.
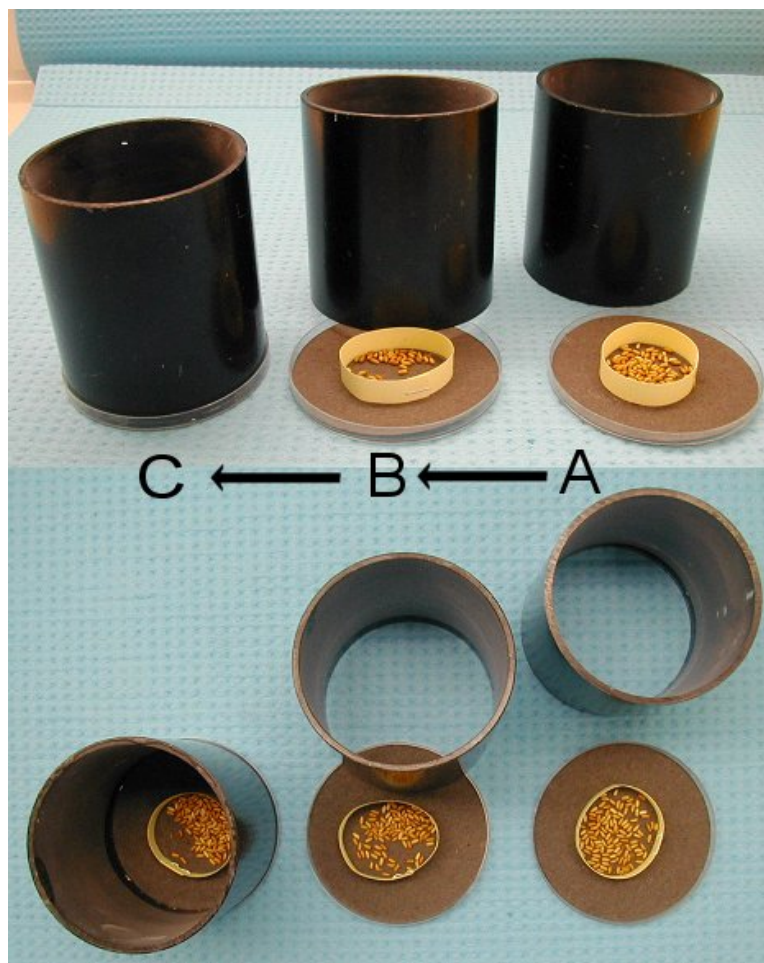
Figure 2.3: Setting up the emergence and flight ability test.

Flies that emerge must be removed from the vicinity of the tubes to minimize fly-back (or fall-back) into the tubes. There are a number of ways to achieve this:

- tubes are placed in a ventilated Plexiglas arena (such as a 30 x 40 x 30 cm ventilated cage) and all flies which have escaped from the tubes are aspirated from the cage once or twice daily.
- flies are allowed to emerge freely into a small room or walk-in screen cage (indoors), and sticky traps or black-light electrocution traps are used to remove live flies.
- tubes are "capped" with a Petri dish lid as soon as it is obvious that emergence is complete, or on the day that the batch would have been released. This is the most appropriate procedure when the test is performed at the fly emergence and release facility.

These or other methods will be suitable, so long as the test conditions can be met and fly-back of weakened flies into flight ability tubes has been demonstrated to be minimal. "Dummy" flight tubes (without pupae, but otherwise similar to tubes used in testing) can be used periodically to estimate the incidence of fly-back. Food and/or water should not be used to lure the flies out of the tubes.

Table 2.2: Specifications for percentages of pupae producing adult flies (emergence) and flies capable of flight (flight ability) for tephritid flies produced for SIT programmes

Species/strain	Minimum			Acceptable mean		
	Pre-irradiation	Post-irradiation	Post-shipment	Pre-irradiation	Post-irradiation	Post-shipment
Emergence (%)						
Ceratitis capitata genetic sexing strain (tsl)	75	70	70	80	75	70
Anastrepha ludens	91	90	80	93	92	86
Anastrepha obliqua	92	90	86	94	92	89
Anastrepha suspensa	85	80	75	93	90	88
Zeugodacus cucurbitae	90	80	70	92	90	85
Bactrocera dorsalis	82	74	70	90	79	75
Bactrocera oleae	80	75	55	85	80	60
Bactrocera tryoni	80	70	65	85	80	75

Species/strain	Minimum			Acceptable mean		
	Pre-irradiation	Post-irradiation	Post-shipment	Pre-irradiation	Post-irradiation	Post-shipment
Fliers (%)						
Ceratitis capitata genetic sexing strain (tsl)	70	65	65	75	70	65
Anastrepha ludens	88	86	71	90	89	79
Anastrepha obliqua	89	85	77	90	88	80
Anastrepha suspensa	75	70	65	85	80	75
Zeugodacus cucurbitae	80	75	-	85	83	-
Bactrocera dorsalis	75	69	62	83	77	72
Bactrocera oleae	70	65	50	77	72	55
Bactrocera tryoni	70	65	60	75	70	65

After emerged flies have flown from the tubes or died, the contents (flies and unmerged pupae) are counted. Percentage emergence and percentage flight ability are calculated as per the formulae in 9.2 Emergence and Flight Ability Assessment Form. The specifications for emergence and flight ability of fruit fly species assessed before and after irradiation and on arrival at the emergence and release facility are listed in **Table 2.2**.

Interpretation

The two most important parameters are percentage emergence and percentage fliers. Additional information (partially emerged flies, deformed flies, rate of fliers), may be useful to the rearing staff. Comparative analyses between data generated at the production facility and the must be performed weekly. High degree of variability or consistent downward trend can indicate an emerging problem that requires corrective action in rearing, handling, shipment methods or materials.

2.2.3. Survival under Stress

Objective

This test is a relative measure of reserves available to the adult fly at different critical periods: at emergence, post-handling/chilling, and post-release.

Discussion

The ability of flies to survive under stress, without food or water, is important in an SIT programme because released sterile insects must survive long enough to become sexually mature and seek mates. Some facilities test survival of flies individually in small cells and have reported better survivorship than when the test is conducted with many flies in a Petri dish as described in the procedure. However, the ability to survive the stress caused by crowding in the pre-release containers is important; hence the standard test requires that the flies be tested in Petri dishes.

This test is conducted at the rearing facility on non-irradiated flies. For comparative purposes flies are also tested post-irradiation, and a further three times at the fly emergence and release facility: on arrival, post-handling/chilling and post-release.

Equipment

- Large Petri dishes (150 mm in diameter) with an opening of approximately 100 mm in the centre of the lid which is fitted with a fine screen (16 mesh screen or 36 squares/cm^2) for ventilation. A hole with a dental wick or small stopper in the side of the Petri dish will allow dead flies to be removed.
- Aspirator or suction pump.
- Environmentally controlled space, no light.
- Emergence cage which allows easy access to remove the flies.

Test Conditions

- Temperature $25 \pm 1°$ C
- Humidity   60-70% RH
- Light   in darkness

Test Frequency

This is a long test, requiring 4 to 5 days to complete from set up. It may be appropriate to conduct it once a week or once a fortnight. Survival is related to pupal weight so an earlier indication of potential survival may be gained from the pupal weight test.

Procedure

A sample of several thousand pupae is placed in an emergence cage without food or water. Within two hours of emergence, 50 males and 50 females (or 100 flies from a production lot if a genetic sexing strain is used) are transferred using an aspirator, or preferably a suction pump, to each of five Petri dishes without food or water. The dishes are held in darkness until the end of the test, the duration of which varies with species (see **Table 2.3**). After the pre-set time, dead flies are removed by tilting the Petri dish, removing the stopper and shaking out the dead flies, taking care not to allow live flies to escape. Both dead and live flies are recorded by sex. Results are expressed as percentage survival. A sample recording sheet is at 9.3 Survival Under Stress Assessment Form.

A similar procedure is followed when the pupae arrive at the emergence and release facility (see **Section 5.2.3**). When the test is performed post-handling/chilling and post-release, samples of flies are collected after the chilling process and from the release bags or boxes.

Table 2.3: Specifications for survival under stress test for tephritid flies produced for SIT programmes.

Species	Test duration (h)	Minimum survival (%)
Ceratitis capitata		
genetic sexing strain (tsl)	48	65
Anastrepha ludens	72	55
Anastrepha obliqua	48	40
Anastrepha suspensa	48	n/a
Zeugodacus cucurbitae	n/a	n/a
Bactrocera dorsalis	n/a	n/a
Bactrocera oleae	n/a	n/a
Bactrocera tryoni	n/a	n/a

(n/a): no data available at present, readers are encouraged to submit data for inclusion in future revisions.

Interpretation

The results of the stress test are generally interpreted as a relative measure of the stored nutrients (food reserves) and water found in the mass-reared adult flies. This measurement is an indicator of the overall elements associated with the larval rearing process, nutritional content of diet, density of larvae per gram of diet in the larval tray, environmental controls and other factors that may affect the insect's ability to store fat reserves through the larval and pupal stage and thus sustain the adult fly. Additionally, when applied at the post-handling/chilling and post-release stages it enables an assessment to be made of the reserves taken up by the fly during feeding at the fly emergence and release facility. Values lower than the standards listed in **Table 2.3** may indicate problems within this process.

2.3. Quality Control Tests Post-irradiation

2.3.1. Radiation Indicator

To ensure that irradiation has been performed correctly, a radiation indicator of the appropriate rating for the dose being administered must be attached to each bag or container of pupae before irradiation. This indicator must be checked immediately on removal from the irradiator. The indicator colour will change from red to black when the specified dose has been administered (**Figure 2.4**).

Figure 2.4: Radiation sensitive indicators before (top) and after (bottom) exposure at dose>125Gy (© ISP 2002).

2.3.2. Emergence and Flight Ability

This test is repeated on samples of pupae after irradiation to assess the effect of the irradiation. The procedure is the same as in Section 2.2.2. Acceptable standard values for emergence and flight ability post-irradiation are in **Table 2.2**.

2.3.3. Survival under Stress

This test is the same as described in Section 2.2.3, using pupae which have been irradiated.

2.3.4. Sex Ratio and Timing of Emergence

Objective

To determine the ratio of males to females within a batch of mass-reared flies. Timing of emergence is used to measure the uniformity of age within a batch of pupae.

Discussion

Significant deviation from a colony's expected sex ratio may give an early indication of rearing problems. These problems could be genetic in nature or stem from procedural effects such as incorrect pupal holding conditions. Monitoring sex ratios becomes especially critical when dealing with genetic sexing strains because sex ratios change with genetic recombination.

Equipment

- Screened emergence grids with 100 individual cells. These are constructed from the open plastic grids that are often used under fluorescent or LED light tubes. The grids are 1 cm high and the cells, open top and bottom, are 1.5 cm square. The grids are cut into sections that are 10 cells by 10 cells (15 x 15 cm), and screening is glued to one side. For larger flies such as *Anastrepha* spp., the grid cells measure 1.8 x 1.8 cm (18 x 18 cm). A sheet of Plexiglas cut to size is used to cover the open side of the grid and is held in place with a large rubber band.
- Manual counter.
- Soft forceps.

Test Conditions

Conditions for holding grids (temperature, humidity, light intensity and photoperiod) should be comparable to those used for fly emergence at the fly emergence and release facility.

Test Frequency

This test should be performed for each shipment, or at least once a week, to ensure that the pupae are being irradiated at the appropriate age.

Procedure

For rearing facilities, this test is initiated at the time of irradiation; for emergence and release facilities, the test is started when hypoxia is broken. For testing, one pupa is placed into each cell and the grid covered with the Plexiglas sheet. Grids are examined twice daily at approximately 8 hour intervals, preferably in the early morning and afternoon. More frequent checks are an option and will provide a more precise estimate of the timing of emergence. The time of the checks should be consistent from day to day at a given facility. At each check, the numbers of emerged males and females are counted. Once it has been determined that no further emergence will occur, the test is terminated.

Results of the sex ratio are expressed as percentage males. Results of the timing of emergence are charted separately by sex as the number of emerged flies over time (normally over a 72 hour period). A sample recording sheet is at section 9.4 Sex Ratio and Timing of Emergence Assessment Form.

Interpretation

The percentage of males in production lots should be within the range of 45-55% for bisexual strains. Production lots of Mediterranean fruit fy *tsl* genetic sexing strains should be over 99% male or over 95% male for other genetic sexing strains based on pupal colour dimorphisms. Sex ratios that deviate beyond the established standards may indicate genetic or processing problems and should initiate a review of each of these components in the mass production process. Some production processes may tend to skew sex ratios, and this needs to be taken into consideration.

Emergence should occur between 24 and 72 hours of irradiation or the break of hypoxia, with a sharp peak of emergence near 48 hours. Any significant deviation from this interval indicates that the timing of irradiation was not optimal. The optimal time for pupae irradiation

is determined by observing eye colour as an indication of physiological age (see Section 3.1 Development stage/age of insects). The presence of more than one distinct peak of emergence when charted indicates the batch of pupae lacks uniformity in age.

2.4. Relevant Literature

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3. Irradiation

3.1. Development Stage/Age of Insects

Objective

To determine the timing of irradiation to enable the release of males that are sterile but of good quality and produce an acceptable sexual performance.

Discussion

Applying the SIT to fruit flies involves irradiation during a narrow time window at the late pupal or early imaginal stage to inhibit reproduction without affecting reproductive capacity, and then release into the target area where the sterile insects compete reproductively with their wild counterparts. Those insects should be irradiated at a specific age to maximize the induction of sterility and to minimize the negative effects on the quality and mating competitiveness of the released sterile adults. This data can be used at mass-rearing facilities to manage pupal-holding conditions and as indicators for optimizing the timing of irradiation.

Equipment

- Irradiation equipment
- Stereo microscope
- Munsell Soil Color Charts
- Dissection instruments

Procedure

A sample of 10 pupae per batch per day are dissected to observe changes in pupal eye colour. The shell of the anterior part of the puparium is carefully removed to expose the eyes of the developing imago. The daily eye colour data is recorded, and then matched and tabulated with the colour scale of the Munsell Soil Color Charts.

Interpretation

The progression of pupal eye colour changes determined for each species can be used as baseline information at mass-rearing facilities for timing the sterilization of pupae at desired holding temperatures, and also for managing pupal development according to programme needs for sterile flies. As example the development of eye colour in *Ceratitis capitata* males (VIENNA-8 genetic sexing strain) at 20°C are presented at **Figure 3.1**. Pupal development time in days until adult emergence of various tephritid fruit fly species and strains at various temperatures is also presented in **Table 3.1**.
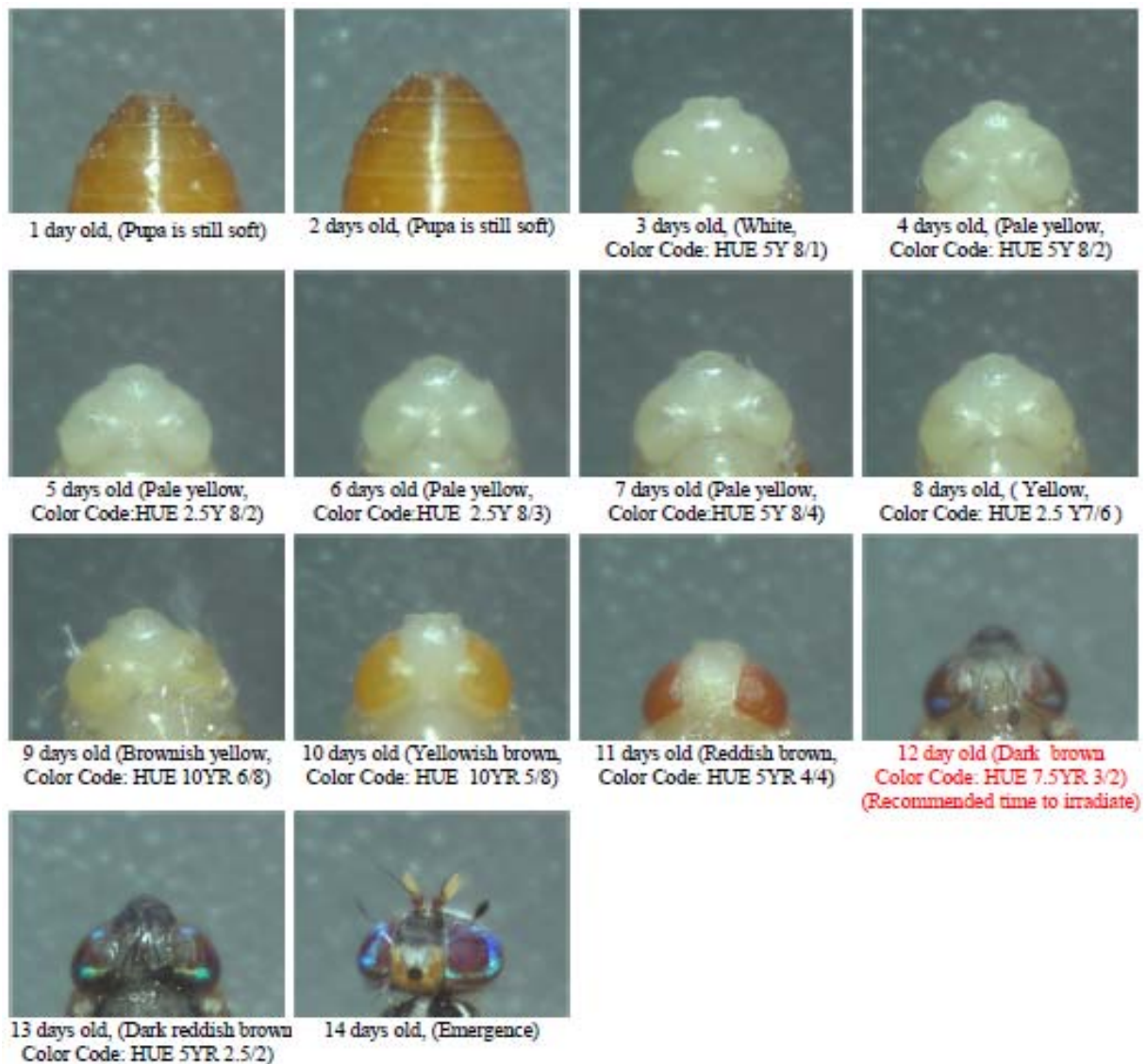
Figure 3.1. Example of eye colour change inside the puparium between pupation and adult emergence of *Ceratitis capitata* flies (VIENNA-8 genetic sexing strain) at 20°C (for denomination of colour and code refer to the Munsell Soil Color Charts (Anonymous, 2000)).

3.2. Pre-irradiation Procedures

Marking of Insects

For SIT programmes (including all that are run in areas where the target pest is not considered to be well established), pupae are marked with fluorescent dye before irradiation. This is done by gently rotating the pupae in a drum or other large container with Day-Glo® powder (1.5 g of powder per litre of pupae, in the case of *C. capitata*) until pupae are uniformly covered with the powder.

Table 3.1. Pupal development time (d) until adult emergence of various tephritid fruit fly species and strains at various temperatures.

Species	Pupal-holding temperature (°C)					
	15	17	20	26	28	20–35[3]
Anastrepha fraterculus (ARG)	57	25	22	14	13	21
Anastrepha ludens	49	-	33[1]	16	15	16
Anastrepha obliqua	40	-	31[1]	14	12	14
Anastrepha serpentina	49	-	31[1]	16	14	15
Zeugodacus cucurbitae (bisexual strain)	45	17	15	10	9	11
Zeugodacus cucurbitae (*wp*-GSS MAR)	-	-	21[1]	11[2]	9	10
Zeugodacus cucurbitae (*wp*-GSS)	-	19	15	9	8	12
Bactrocera dorsalis (bisexual strain)	-	19	16	10	9	12
Bactrocera dorsalis (*wp*-GSS)	-	19	16	10	9	16
Bactrocera dorsalis (African population)	-	21	16	11	9	13
Bactrocera oleae	44	19	15	11	10	17
Bactrocera tryoni	-	-	18	11	9	13
Bactrocera zonata	-	-	21[1]	11[2]	9	10
Ceratitis capitata (bisexual strain)	-	17	16	10	9	11
Ceratitis capitata (VIENNA-8)	-	17	14	9	8	15
Ceratitis capitata (VIENNA-8 SAF)	29	-	18[1]	11[2]	9	-

[1]19°C

[2]25°C

[3]Natural environment (temperature fluctuated between 20 and 35°C)

Packaging for Irradiation

Container:

The size and shape of the packaging container are typically a function of the size and shape of the chamber in the irradiator.

Polyethylene bags. Pupae are sealed within polyethylene bags or "sausages" that are ca 1.5 MIL thick. (MIL is one thousandth of an inch = 0.0254 mm). In some cases, pupae are double bagged (before irradiation) as an extra security measure to prevent bags from breaking.

In case of Gammacell irradiators (for example at El Pino, Guatemala and Arica, Chile) the canisters accept plastic bags containing a maximum of 2 litres of pupae.

In Case of Cobalt-60 484 (for example at El Pino, Guatemala) the canisters accept plastic bags containing a maximum of 20 litres of pupae.

In the case of Husman irradiators (for example at the USDA Mexfly facility, USDA Hawaii Fruit Fly Facility and El Pino) the canisters hold longer plastic "sausage" bags that contain 4 litres of pupae each.

Hypoxia:

Use of reduced-oxygen atmospheres during irradiation of tephritid pupae is mandatory to reduce the generation of free radicals by gamma or X rays that induce collateral intracellular damage during irradiation, thereby allowing higher levels of sterility to be attained without unduly reducing the quality and competitiveness of the resulting flies. Typically, pupae are sealed in the airtight packaging container and held at cool temperature (12 - 20° C) for at least 1 hour before being irradiated. During that period, the insects exhaust most of the oxygen supply within the container. If containers are rigid (e.g., bottles), they should be filled to near capacity to minimize the amount of excess air. If plastic bags are used, they should be tied tightly after excess air has been expelled. An alternative method of achieving hypoxia is to saturate the atmosphere within the container with an oxygen-free substitute for air (e.g., N_2) but increased radiation doses are required to achieve similar sterility level compared with hypoxia conditions. Cool temperatures and hypoxia are necessary to reduce the metabolic rate of pupae during irradiation and subsequent shipment. Hypoxia however is not without effect on the quality of the flies, and **Table 3.2** illustrates the effect of hypoxia over varying periods.

Table 3.2: Emergence and flier data for fruit flies exposed to post-irradiation hypoxia for different periods of time.

QC Centre/Species	Duration of hypoxia(h)	Emergence (%)	Fliers (%)
Flies reared and tested at the USDA Hawaii Fruit Fly Rearing Facility			
Ceratitis capitata	0	95.6	92.5
(unirradiated)	1	94.1	90.3
	10	94.3	89.2
	20	94.1	88.9
	30	94.1	85.8
	45	92.9	82.2
Flies reared and tested at the Moscafrut, Mexico Fruit Fly Rearing Facility			
Anastrepha ludens	0	93	90
	15	86	81
	25	85	80
	35	84	78
	45	81	74
Flies reared and tested at the Moscafrut, Mexico Fruit Fly Rearing Facility			
Anastrepha obliqua	0	92	87
	15	89	79
	25	88	78
	35	83	75
	45	79	74
	55	75	66

Selection of Absorbed Dose

The radiation-absorbed dose (hereafter dose) that is used to induce sterility is of critical importance to SIT programmes. The methods for measuring radiation dose are given in chapter 4 (Dosimetry). Insects that receive too low a dose retain too much fertility for programme purposes and, in some instances, could even compromise quarantine security. Too high a dose will result in insects that do not compete well against wild flies in the field, reducing the effectiveness of the SIT programme. The balance between sterility and quality is an important one.

The dose to be used for any given SIT programme should be based on mutual agreement between the rearing facility and the end user. The decision is based on the results of the Sterility Test (section 7.1) in combination with such considerations as programme requirements and effects of radiation dose on insect quality. Typically, the programme manager will want to specify an optimum dose range to achieve appropriate levels of quality and sterility or a *minimum dose* that all pupae must receive, but often this becomes a political decision and too high a dose is prescribed. Additional information can be found in the Directory of SIT Facilities (DIR-SIT, http://nucleus.iaea.org/sites/naipc/dirsit/SitePages/Home.aspx) for the current doses used.

In most tephritid flies, the dose required to stop egg production in females is typically lower than the dose that is required to induce near-complete sterility in males. For most purposes, the *minimum dose* will be somewhat higher than the dose at which egg production stops.

Dose Mapping and Loading Configuration

The dose rate will vary somewhat within the irradiation chamber. To ensure that all pupae receive a *minimum dose*, most pupae will actually be irradiated with a dose that is somewhat higher than the specified minimum. If the dose-uniformity ratio (ratio of maximum to minimum dose rate within the chamber; see SOP, [FAO/IAEA 2013a, b]) is too high, many of the insects will end up receiving a dose that significantly reduces their competitiveness. In such cases, it may be necessary to use only portions of the irradiation chamber where the dose rate is more uniform. For example, plugs of Styrofoam or material of similar density could be used to fill parts of a chamber if the dose rates in those areas are found to differ substantially from those in more central portions of the irradiation chamber.

The size and shape of the container used to hold insects during routine irradiation processing, its position within the canister (if applicable), and other relevant information (e.g., any plugs that are used to exclude samples from portions of the canister where the dose rate is inappropriate) should be defined for each facility according to the dose range that will be used.

3.3. Irradiation and Process Control

Irradiation Procedure

The irradiation facility and the procedure used to irradiate insects must be thoroughly characterised and tested to ensure with a high degree of confidence that the process will properly sterilize the insects. Methods used in characterising the irradiation facility itself are covered by ASTM guidelines (ASTM 2002a) and the method for dosimetry is given in chapter 4. For each insect type and for each irradiator, the dose mapping discussed above will help establish the process parameters needed to deliver the correct dose range.

Process Control

Accidental release of flies that are not irradiated properly could be potentially disastrous, especially for programmes where SIT is used for eradication of extremely small populations and/or as a prophylactic measure to prevent establishment of newly introduced flies (for example in the preventive release programmes in California and Florida). The following process control procedures are essential to minimize the chances of such accidents.

a) *Dosimetry and sterility tests:*

Dosimetry and sterility tests should be routinely performed (ideally for every shipment) to ensure that the irradiation process used delivers the expected dose and results in the desired level of sterility. The procedure for dosimetry at a radiation facility for SIT is thoroughly described in SOP (FAO/IAEA 2013a, b), while the sterility testing procedures are described in 7.1 Sterility test. In addition to dosimetry and sterility tests, relevant operating parameters of the irradiator (e.g., timer setting and position of the canisters) should be monitored and documented.

b) *Radiation sensitive indicators:*

A radiation-sensitive indicator is a material such as a coated or impregnated adhesive-back (or adhesive-front) substrate, ink, or coating which may be affixed to or printed on the container and which undergoes a visual change when exposed to ionising radiation ISO/ASTM Standard 51539:2013. These indicators are designed to be dose-specific; i.e., they indicate that they have been exposed to a minimum level of radiation (see **Figure 2.4,** section 2.3.1). Experts in dosimetry agree that these indicators can provide a visual and qualitative indication that a sample has or has not been exposed to a pre-defined absorbed dose. However, the indicators cannot be used as a substitute for proper dosimetry. Radiation-sensitive indicators must be handled and stored according to their manufacturers' recommendations. Indicators that are exposed to excessive humidity, high temperature or UV radiation (e.g., sunlight) before or after irradiation may give erroneous readings.

A radiation-sensitive indicator should be firmly affixed to each packaging container of insects before the insects are transported into the room where they are to be placed in the irradiator. If insects are packaged in plastic bags, the indicator should be placed *inside* (but visible from the outside, see **Figure 3.2**) or between double polyethylene bags. Indicators can be purchased with a sticky front surface so they can be affixed to the inside of the bag and viewed readily both before and after irradiation.

Figure 3.2: Polyethylene bag containing sterile Mediterranean fruit fly pupae shipped from Guatemala (USDA-Moscamed rearing facility) and opened at the emergence centre in Israel (The sign "OK" on the indicator is hand-made by the QC officer at the irradiation facility, after irradiation and before shipping).

The absorbed dose at which the indicator "changes" should be below but near the minimum dose that is used for processing the insects. Indicators should be examined before use and discarded if they show any sign that they have been inadvertently exposed to radiation. New batches of indicators should be checked to ensure that their sensitivity agrees with the specified dose.

c) *Administrative control:*

The administrative procedures described here are applicable to sterile insect programmes in areas where the target species is not considered to be established within the release zone. Slightly less stringent versions of these procedures *may* be allowed in some suppression programmes that are being conducted against well-established populations.

Securing the irradiation process. Every insect sterilization facility must have a Radiological Safety Manual officially approved by the respective National Radiation Protection Authorities (NRPA). The person responsible for the facility radiological safety and the facility operator must be accredited by the NRPA and a valid license for operations must be held. The license should be periodically renewed as required by the respective NRPA. Before initiation of the irradiation activity, the authorized operator must ensure that all safety measures are in place and working properly. Before initiating the irradiation procedure, the operator must record the conditions of the irradiation facility in a log-book. Any irregularity in the functioning of the

irradiator should be immediately notified to the person responsible for radiological safety and the process cancelled until further notice. The operator must, at all times, observe safety regulations following the Radiological Safety Manual of the respective facility.

Separation of processed containers. Insects should be securely sealed in their packaging containers in an area separate from both the irradiator and the boxing/loading areas. Ideally, entry and exit openings for the irradiator should only be accessible from different rooms to avoid possible mix-ups of irradiated and unirradiated containers of pupae (this is clearly not possible with irradiators where samples have to be inserted into and removed from the irradiator through a single opening). Packaging containers are not to be broken or opened from the time they are sealed for hypoxia (pre-irradiation) until they reach the emergence and release facility.

Verification at the production facility. Before irradiated packaging containers of sterile insects are removed from the irradiation facility, the personnel should examine the radiation-sensitive indicator on each container and either provide written certification on the container, as shown in **Figure 3.2**, showing that it received the correct dose or reject the container if the indicator was not fully exposed or no indicator is attached.

Verification at the emergence and release facility. Examine each packaging container (bottle or plastic bag) of pupae. Pupae in the container must be destroyed if:

- the radiation-sensitive indicator is missing, unexposed or partially exposed;
- there is no certification on the packaging container showing that the container was correctly irradiated;
- the packaging container is broken or has been opened.

3.4. Dosimetry

The objective of dosimetry is to provide an accurate estimation of maximum and minimum radiation absorbed dose (hereafter dose) given during the fruit fly pupae sterilization process.

There are various reasons for performing dosimetry depending on the process. For example, there are 'regulated processes' such as food irradiation and sterilisation of health care products, where it is a legal requirement to perform dosimetry. In other cases, like plastic-insulation modification in electrical wires, the product quality and the economy of the process are the driving forces. In some other cases, it helps to scale up a process from the research level to the industrial level. Nearly all of these requirements apply to the case of Sterile Insect Technique (SIT) projects. Species targeted by SIT programmes are typically major pests affecting agriculture or human health, so the assurance that insects have been properly irradiated is of crucial importance to guarantee the efficiency of the SIT. This is achieved through standardized dosimetry, the key element of which is accurate and reliable dose measurement. The principal role of dosimetry is in establishing the required minimum and maximum dose and also ensuring that the correct dose is delivered to each insect container.

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4. Packing and Shipping Procedures

Transboundary shipment of sterile insects has taken place on a continuous basis since the Sterile Insect Technique (SIT) was first developed. By 2001, the total number of sterile insects shipped was estimated at over 960 billion in more than 12,000 shipments to 22 recipient countries from 50 sterile insect production facilities in 25 countries. During a period of almost 50 years, only one problem associated with shipping live sterile insects has been recorded. This case was related with non-irradiated screwworms that were shipped to different locations for release. Human error was the cause of this incident that could have been prevented if standard operation procedures (SOP's) had been observed (see 3.3 Irradiation and Process Control). This single case in thousands of sterile insect shipments carried out throughout more than 50 years, shows that any system is subjected to failure and it illustrates the importance of strict observance of SOP's to mitigate the risk of hazards occurring. In a half a century, and over 450 billion sterile pupae of tephritid fruit fly species (see Appendix D: Transboundary Shipments of Sterile Insects), no shipment of sterile insects has ever been subjected to prohibition by national or international plant protection or regulatory authorities.

The risks from transboundary movement of sterile insects have been determined to be negligible if procedures outlined in this manual are followed. Some countries do not regulate shipment of sterile insects, others only require labelling and documentation, and still others are regulating sterile insects under their biological control measures. With the increase in the number of countries applying SIT and the number of new production sites, this guideline will assist factories or any other organization shipping sterile insects to follow standard operation procedures thus assuring safe shipment while facilitating trade.

4.1. Packaging Procedures

Packaging for Shipping

Size and weight of packages can take many forms depending on type of irradiator used but should be designed to minimize breakage (see **Figure 4.1** for examples).

Polyethylene bags of sterile pupae are loaded into secure cardboard shipping boxes for short and longer distance transportation to fly emergence and release facilities. As an example, the shipping boxes used in the Moscafrut facility in Mexico (**Figure 4.1 c**) to hold the 9-liter bags filled with irradiated pupae are constructed of double-walled corrugated cardboard of 74 x 41 x 34 cm with a top and bottom full overlap. Inside the box, a central compartment, 59 cm long, is lined with additional layers of corrugated cardboard. The nine bags of pupae are placed lengthwise within this central compartment in three layers of three bags each. Layers, as well as bags within a layer, are separated by spacers of double and single-wall, respectively, corrugated cardboard. The space remaining at either end of the box (≈10 cm of the length of the box) is used to hold two packages each of frozen "blue ice", wrapped in newspaper) (**Figure 4.1, a, b**).

Figure 4.1: Inside views of boxes (a, b) used to ship sterile Mediterranean fruit fly pupae from Guatemala (USDA-Moscamed rearing facility), and sterile mexfly pupa (c) from Tapachula Mexico, to the north of the country.

In some instances each bag with sterilized pupae contains and irradiation indicator label, while in others the entire shipping box is irradiated. This allows the placement of the indicator label where it can be viewed externally. As example see **Figure 4.2**. However, the label colour can change when exposed directly to sunlight, excess humidity and high temperature.

Once full, a box is sealed closed with carton staples (placing staples in locations where they will not hit the bags of pupae) and two bands of fibre-reinforced plastic adhesive tape (**Figure 4.2**).

Figure 4.2: Boxes used for shipping sterile mexfly pupae from Tapachula Mex, (Moscafrut rearing facility).

Labelling

All boxes are properly labelled with the words: "Fragile" and "Biological Material". In some cases, the mention "Live Insects" and some indication of the storage conditions ("This Side Up", "Handle with Care" "Do not stack more than 3 boxes") are also present on the boxes (see **Figures 4.3)**. As noted below (see 4.2: Shipping and Handling Procedures), the boxes should not be held at temperatures below 18-20°C.
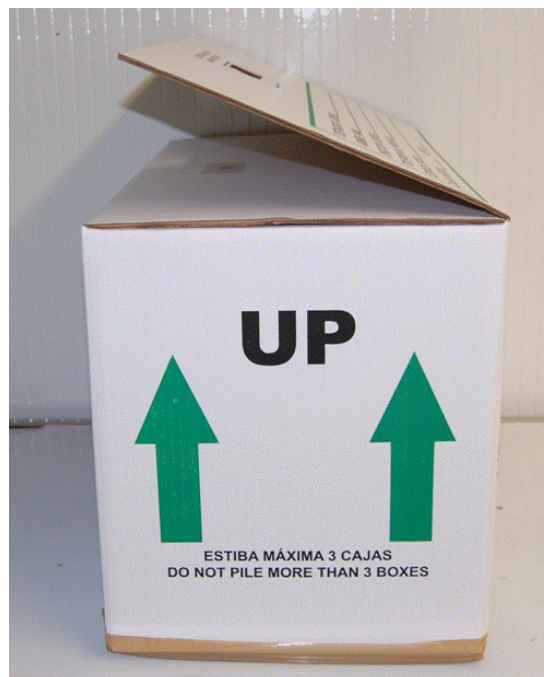
Figure 4.3: Labels placed on boxes containing sterile mexfly pupae shipped from Tapachula (Moscafrut rearing facility) within Mexico, or to California USA.

To facilitate tracking of consignments, these should have complete information on the location of the addressee, a shipment number and in addition, boxes for each shipment have to be numbered consecutively in large, clear writing on the outside of the box; e.g., "Shipment 2729, Box 3 of 14".

4.2. Shipping and Handling Procedures

Boxes should not be handled abruptly or be subjected to inadequate piling and compacting to prevent accumulation of unwanted levels of metabolic heat. Irradiated pupae are also sensitive to excessive vibration which could cause high levels of insect mortality. Prior to shipping and during transit, sealed boxes should be placed in close and clean facilities to prevent carrying contaminating pests in shipments (hitch-hikers).

Monitoring Shipping Conditions

Boxes of pupae should be held at or slightly below 20° C during transportation. In all cases, the containers must not be held under freezing conditions or spend more than a few minutes at temperatures above 30° C, or under conditions, such as prolonged exposure to direct sunlight that would create internal temperatures above 30° C.

Ideally, data loggers should be placed inside the containers in order to monitor temperatures inside and outside cardboard boxes during transport, since drastic variations could affect the overall quality of the emerged flies, but in specific the timing of emergence, and in consequence the of age released insects. In **Figure 4.4** we can be observe temperature variations (both, inside and outside boxes) in some shipments from Guatemala to Florida USA.
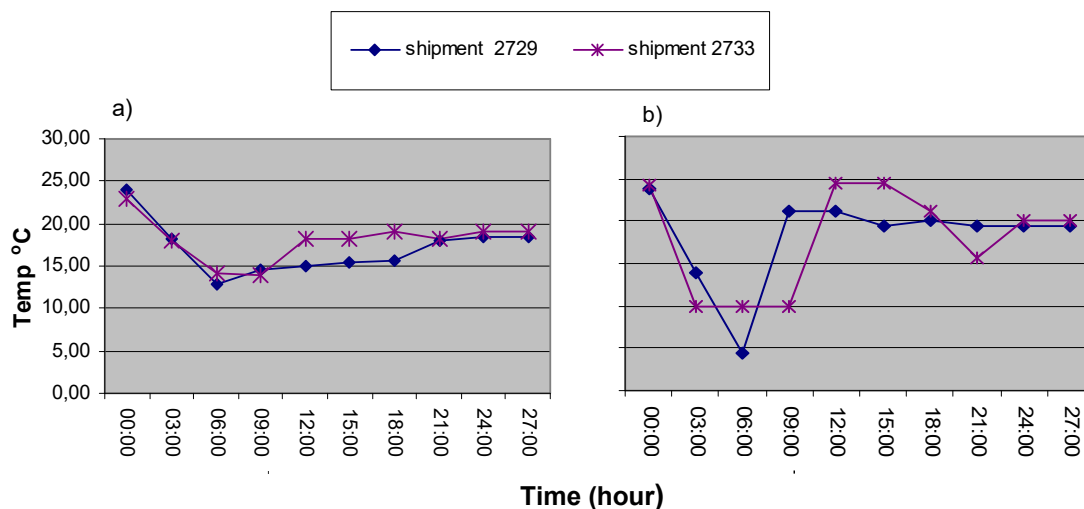
Figure 4.4 Temperature record in two shipments of sterile male of *C. capitata* from Guatemala to Florida: a) inside the box, b) outside the box.

For local transportation, air-conditioned or refrigerated vans should be used if ambient conditions are likely to result in overheating of pupae. For long-distance shipment, pupae are typically carried by commercial airlines in a portion of the cargo hold where temperature and air pressure are held at "cabin" levels. Airline routing should be selected to minimize transhipment points and overall shipment time. Although pupae have been held under hypoxia for 40 hours for some programmes, quality begins to drop rapidly when hypoxia extends beyond ≈24 hours.

The supervisor of packing and shipping should complete a data-sheet with the specifications and conditions of the sterile pupae being shipped. The datasheet should be signed by the

supervisor and a copy should always accompany the consignment. The supervisor should also file a copy of each of the documents (see 4.3 Shipping Documents) which accompany the consignment regardless of the destination (i.e., national or international).

Upon arrival at final destination and after the consignment has been cleared by the national phytosanitary and customs authorities, the receiver must carefully check the datasheet that accompanies the consignment and verify: (i) that the datasheet has been signed by the shipper, and (ii) that the content of the package matches the information reported on the datasheet. Of particular importance is verifying the condition of the irradiation indicators attached to each pupae container. The indicators must clearly show that they have been exposed to the specified absorbed irradiation dose as explained in 3.3: Irradiation and Process Control. In addition to this irradiation indicator label, it is recommended that each box be stamped and signed by the facility personnel overseeing the irradiation and shipping process. (**Figure 2.6**). The receiver must then sign a statement that the product has been received according to specifications. Any discrepancy on the consignment content should immediately be reported to the shipper and a decision on keeping or discarding the consignment should be made. Any visual sign on the indicators of inadequate pupae irradiation is sufficient to dispose, in a safely manner, the whole consignment content.

4.3. Shipping Documents

Packages should be accompanied by the necessary documentation to guarantee timely and safe delivery. Shippers should be vigilant of the following:

- Documentation should conform: (i) to relevant regulations of exporting and importing countries, especially concerning import permit, national transit permit, phytosanitary certificate, irradiation certificate, labelling and notification, and (ii) to transit regulations should the shipment transit through a third country (i.e., a country that is neither the country of origin nor the country of destination of the consignment) (**Figure 4.5**).
- Documents should include clear instructions to handlers and officials at the point of embarkment, transhipment and entry on how the package should be treated to avoid damage to the contents and on action to be taken if the package is breached.
- The documentation should indicate that package content is perishable and therefore rapid transit of the material should be allowed.
- The receiver should have the necessary documentation to provide rapid feedback when the package is delayed.
- The receiver might request data on the quality of the sterile insects being reared
- The receiver should request, for each consignment, a datasheet with a minimum of information.
- Documents should also include clear instructions to officials at transhipment or entry points on how a lost package that is found is to be discarded.

Figure 4.5: "Transit" documents for shipment of sterile mexfly pupae from Tapachula Mexico to the north of the country or California USA.

4.4. Relevant Literature

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5. Routine quality control tests at fly emergence and release facilities

Fly emergence and release facilities are responsible for the final steps of an effective field use of the sterile insect technique (SIT). The correct handling of sterile insects (SI) at this stage is crucial to deliver to the field, sexually mature, robust and sufficient SI to conduct the required pest population control.

To ensure that the procedures followed during the shipping and handling will render SI in the desired condition, a series of tests that document SI performance are recommended. These tests document, at each step of the process, the quality of SI before and after each of the handling phases that occur in a fly emergence and release facilities. The tests should be conducted at the following stages:

- Arrival of the SI shipment;
- After the handling and chilling process;
- After the release of SI in the field.

All of these evaluations, when compared, should allow detection and quantification of any losses in SI quality, and at which phase of the process they are occurring. In turn, this would allow commencement of corrective measures. Standardization of these procedures on a global basis also allows information exchange and comparisons of SI performance amongst fly emergence and release facilities

5.1. Guidelines for Sampling Insects for Routine QC Tests

For quality control tests on arrival the same sampling procedure should be used as at the mass-rearing facilities (see 2.1 Guidelines for Sampling Insects for Routine QC Tests). The only difference is the sampling of adults at post-handling/chilling and post-release, but this is addressed under each test procedure (absolute fliers and survival under stress).

Provided that handling procedures at fly emergence and release facilities are followed as described in the existing manuals (i.e., Guidance for packing, shipping, holding and release of sterile flies in area-wide fruit fly control programmes FAO/IAEA, 2007; Moscafrut Manual 2010; USDA-APHIS 2008), major deviations in insect quality should not be observed.

Changes to handling procedures at fly emergence and release facilities, such as holding density inside rearing containers (PARCS and/or Towers), temperature and humidity regimes and types of food, that could be introduced should be properly validated to maintain the sterile fly quality.

5.2. Quality Control Tests on Arrival

Evaluation of sterile insect quality on arrival at the fly emergence and release facilities is crucial to corroborate that the shipping conditions combined with the quality of the insect are adequate for their additional handling and eventual release. Accurate measures that allow comparisons between shipping and receipt are therefore important. For comparison purposes,

measuring equipment should be calibrated, test procedures should be similar to those used at mass-rearing facilities, and age of insects should be standardised.

The comparative evaluations of insect quality are represented by the following tests:

- Radiation indicator
- Pupal weight
- Emergence and flight ability
- Survival under stress
- Sex ratio and timing of emergence

5.2.1. Radiation indicator

Upon arrival of the sterile insect shipment to the fly emergence and release facilities, a careful examination of the radiation indicator label should be conducted, to confirm that the label has changed colour (**Figure 2.6**). If there is no evidence that the colour has changed, the entire package should be destroyed.

5.2.2. Pupal weight

Objective

To determine the pupal weight of sterile insects at time of their arrival at fly emergence and release facilities and measure the effect of packing and shipping.

Discussion

A compilation for the different fruit fly species of what is currently considered an appropriate pupae weight is presented in **Table 2.1,** see 2.1.1 Pupal Weight. Pupal weight, as determined by the fly emergence and release facilities at arrival, is a direct comparison with the value determined when the pupae leave the rearing facility. Pupal weight reduces as adult emergence is approached (Langley 1970; Nestel et al. 2003), but there should be minimal weight reduction between departure from the mass-rearing facility and arrival at emergence and release facilities.

Equipment and Procedures

This test should be carried out at the fly emergence and release facilities exactly as it is conducted at the mass-rearing facility (see 2.1.1 Pupal Weight).

5.2.3. Emergence and flight ability

Objective

To obtain an accurate estimate of the percentage of pupae received by the emergence and release facilities that will emerge and produce adults that are able to fly.

Discussion

Fly emergence and release facilities receive pupal shipments that originate at different distances from where the fly emergence and release facility is located. To ensure that the appropriate handling and shipping conditions are used to deliver the sterile insects, fly emergence and release facilities should measure percentage of emergence and percentage of adults that are able to fly. The results of these evaluations should be compared to the results obtained at the mass-rearing facility prior shipment (see 2.2.2 and 2.3.2).

Equipment and test conditions

To obtain estimates of the percentage of emergence and percentage of adults that are able to fly, the same equipment and testing conditions should be used as in section 2.2 (see 2.2.2. Emergence and Flight Ability).

Procedures

The main difference between the methods already described on how to perform these tests at the mass-rearing facilities and the fly emergence and release facilities is that the physiological age of pupae at arrival is uncertain (as it has been affected by variation in shipping temperatures and hypoxia). Fly emergence and release facilities should immediately open the containers that transport the pupae (break hypoxia), measure pupal temperature, and prepare the pupae for testing.

Interpretation

Percentage of emergence and percentage of adults that are able to fly reflect whether the conditions for shipping sterile insects to the fly emergence and release facilities were adequate. A long period of hypoxia can have a detrimental effect on these values, reducing them as time in hypoxia increases (FAO/IAEA, 2007). Lower relative humidity at the fly emergence and release facilities can reduce the percentage of emergence and percentage of adults that are able to fly. A compilation for the different fruit fly species of what is currently considered an appropriate percentage of emergence and percentage of adults that are able to fly presented in **Table 2.2,** see 2.2.2 Emergence and Flight Ability.

5.2.4. Survival under stress

Objective

To determine the survival of sterile flies after their mass-rearing, sterilization, packing and shipping procedures.

Procedures

This test should be conducted as described in section 2.2.3 Survival under Stress.

Discussion

This measurement can be compared to the survival of the sterile insects as determined at the mass-rearing facility prior to their departure to the fly emergence and release facilities. This comparison also allows assessment of packing and shipping procedures.

As a consequence of these comparisons, recipients of sterile flies can recommend to the mass-rearing facilities, need for adjustments to their rearing, packing and shipping conditions.

Interpretation

Survival under stress can be affected by conditions during delivery (e.g., temperature, time in hypoxia). If differences in survival under stress are detected between the mass-rearing facility and the fly emergence and release facilities, there is a need to investigate damaging conditions during shipment.

5.2.5. Sex Ratio and Timing of Emergence

Objective

To determine the proportion of sexes (males and females) within a sterile pupae shipment and to determine the number of hours required, after pupae arrival, to reach 50% emergence of adult sterile insects as well as the age of the sterile insects at the time of their release.

Discussion

On arrival of pupae at the fly emergence and release facilities, samples should be taken to determine the sex ratio of the shipped pupae. This test could be combined with the determination of the time of emergence to avoid duplication of effort. For species in which sexing strains are being used, the expected sex ratio is strongly skewed toward the males. Nevertheless, ratios should be determined since an increase in the numbers of released females will lead to increased detections of sterile females in monitoring traps.

Emergence time is related to the age of the pupae at the time of arrival at the emergence and release facilities, time in hypoxia, shipping conditions, and temperature at the emergence and release facility. Determining the time of emergence of sterile insects is important because it also allows calculating the approximate age of the sterile insects at release. This age should be as close as possible to the initiation of mating activity of the sterile insects (e.g. for Mediterranean fruit fly an age of 5 days).

Equipment and Procedures

Equipment and procedures followed to determine the proportion of sexes and timing of emergence are described in section 2.3.4. Sex Ratio and Timing of Emergence.

Interpretation

See section 2.3.4. Sex Ratio and Timing of Emergence. The fly emergence and release facilities managers should be aware that environmental conditions prior to release of insects affect their emergence time and sexual maturation.

5.3. Quality Control Post Handling/Chilling

5.3.1. Adult Fliers

Objective

To determine the percent flight ability of sterile insects after the chilling process.

Discussion

Some fly emergence and release facilities use paper bags or containers to release the sterile insects directly, while some others chill the sterile flies for their collection and release. Sampling emerged and handled flies is different for each of these release systems.

Chilled adult release

In this system sterile insects are exposed to cold temperatures in order to immobilize them to facilitate their handling and eventual field distribution. This process occurs inside cold rooms specially designed to maintain a range of 3-4 ºC (38 ºF). Plastic adult release containers (PARC) and/or emergence towers (ET), containing emerged and fed sterile flies are moved inside cold rooms where the "cold treatment" is provided for a period of about 30 to 60 minutes. Chilled insects are then funnelled to release boxes for their field dispersal. As a measurement of the actual numbers of flies to be released as well as the quality of the processes (handling and chilling and insect quality *per se*) the percentage of adults fliers should be determined.

Equipment

- Same equipment as used for the Emergence and Flight Ability test (section 2.2.2.), however in addition the following is needed:
- 100 ml beakers and lids
- Serving trays
- Freezer
- Entomological forceps (soft)

Procedure

Quality control personnel using beakers randomly collect 5 samples of chilled sterile adults (5 ml for medfly) from each knockdown procedure (volume collected should be adjusted according to the fruit fly species), to collect approximately 100 flies per sample.

Use containers with known tare weight to weigh the 5 adult fly samples. Transfer the samples to individual Petri dishes and place a black tube on top of each Petri dish (as described in section 2.2.2: Emergence and Flight Ability test).

After finishing sample preparation, Petri dishes are transferred to a temperature controlled room at 25 +/- 1 ºC and 65% RH. After a period of several hours (four hours for medfly, see **Figure 5.1**), flight tubes are covered with a lid and are transferred/placed inside a freezer to immobilize the flies for a minimum of 30 min to weight of the remaining insects in the tubes. These times need to be adjusted according to the fly species.
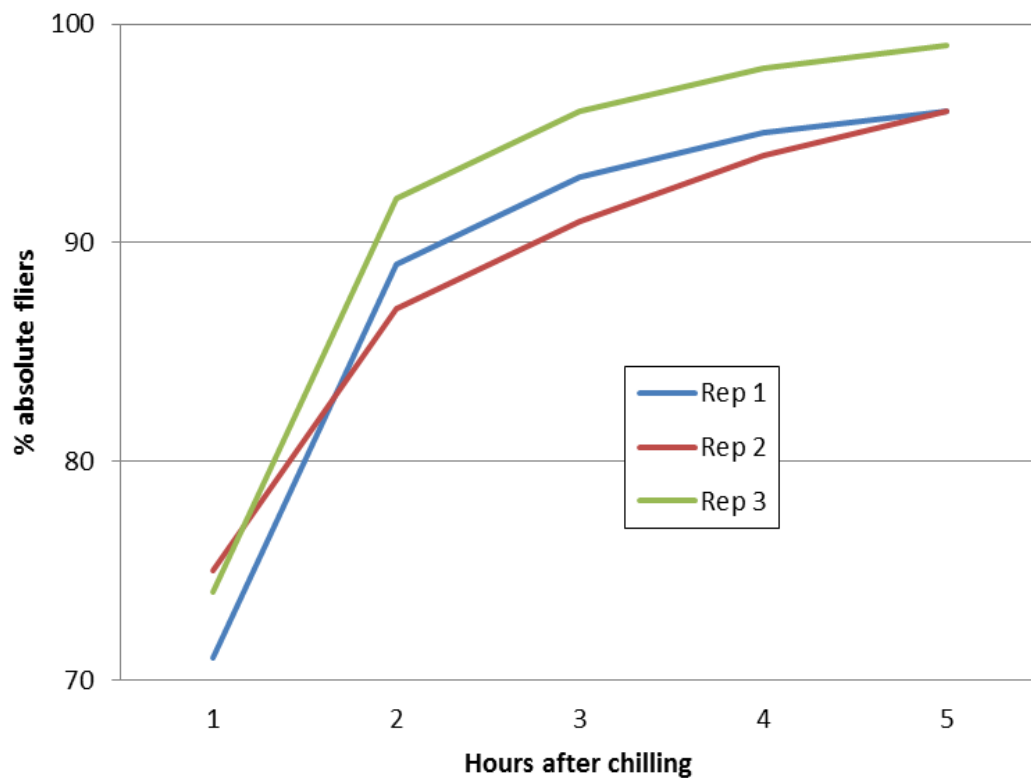
Figure 5.1 Cumulative percentage of *Ceratitis capitata* flies out of the tube (absolute fliers) five hours after chilling.

Adult Fliers are the percentage of the emerged adults able to fly after the process of packing, chilling and release. The value of this parameter is calculated by the difference between the total fliers in the sample and the non-fliers that remained inside flight tubes adjusted by the percent of adult emergence as obtained in the Flight Ability test (5.2.3). The percentage of Adult Fliers (FA) is calculated as follows:

$$AF = [(T+WTF-WNF) / (T+WTF)] \times PE \times 100$$

T = weight of the container (tare).

WTF = weight of the total flies and residues.

WNF = weight of the non-fliers and residues that remained in the container.

PE = Percent of emergence

Additionally, it is important to know the average weight of the individual adult flies after chilling in order to calculate the total number of flies inside the release box.

Interpretation

The availability of sufficient number of sterile insects for field release is insurance for the successful application of the SIT. Handling of insects in the last phases of production, emergence and release becomes a relevant issue. Mismanagement of these later stages could severely reduce the numbers of sterile insects available, making the SIT operation and expensive endeavour. Factors such as humidity and condensation inside the cold rooms and release containers can have a severe impact on final percent fliers, the conditions required for a successful application of the chilled adult technology will vary according to the fly emergence and release facilities location. Managers again should ensure that the conditions are met for optimal sterile flies viability. For users of bag releases, crowding conditions during holding, handling and transportation can have a severe effect in reducing the percentage of adult fliers; operational practices should be validated/questioned prior to full implementation.

5.3.2. Survival chilled/released flies under stress

Objective

This test is a relative measure of reserves available to the adult fly at different critical periods: at emergence, post-handling/chilling, and post-release.

Procedure

The equipment needs and testing procedures for this test are similar to those described in the methodology in section 2.2.3: Survival under Stress. However, the test differs in that adult flies are collected post-handling/chilling. This is done by taking five samples of 20 ml from the chilled adult release box, or from bags, tower levels or PARC boxes that are placed under cold temperatures to be able to handle the flies for the test. The sampled flies are placed inside a 30x30x30 Plexiglas cage; 30 min later, 100 flies are taken randomly using an aspirator and placed inside a Petri dish as described in 2.2.3. Five repetitions must be done.

Interpretation

In addition to what has been stated in section 2.2.3: Survival under Stress, the survival of sterile flies during the fly emergence and release facilities rearing and handling process can be optimized by providing insects with good quality and quantity of adult food. Issues like accessibility of insect to food sources as well as the crowding conditions within the holding containers play an important role in maintaining insect quality. The fly emergence and release facilities must validate their fly emergence and handling protocols to ensure proper conditions for sterile insect during the process.

5.4. Quality Control Post Release

The post-release QC evaluations should be carried out to determine the final quality of sterile flies deployed on the field. The tests should include at least an estimation of the number of absolute fliers as well the percent of survival flies under stress.

Samples of flies to perform these tests must be obtained as under procedures for absolute fliers post chilling (see section 5.3.1 Adult Fliers). The samples, once available, should be placed inside Velcro-bags (see below for details), then situated inside the release machine. This method allows the samples to travel under similar conditions as the flies that are actually released. Upon completion of the flight, retrieve the samples, briefly chill the flies to be able to handle them, and conduct the absolute fliers and survival tests as described.

In the case of the release using paper bags and/or containers that do not require chilling, randomly selected a bag or container mark it and allow to travel under the conditions of the actual release bags/containers. Bring the designated sample back to the facility to conduct these two tests.

5.4.1. Adult Fliers

Objective

To obtain an estimation of percentage of the post-released flies that have the capacity to fly.

Discussion

Sterile flies are exposed to cold temperatures in order to immobilize them to facilitate their handling and eventual field distribution. Chilled flies are then funnelled to airplane boxes for their aerial dispersal. As a measurement of the effect of the chilling and ferry time of aerial deployment on the insect quality *per se*, the percentage of adult fliers post- release needs to be determined.

Equipment

Same equipment as used for Absolute fliers post-chilling (section 5.3.1 Adult Fliers), but in addition, the following equipment is needed:

- Five Velcro bags (net-bags of 15 x 20 cm, 2 faces joined with Velcro; see **Figure 5.2b**).

Procedure

During the knockdown process in cold rooms, 5 samples of 5 ml of chilled flies each are taken randomly and are carefully introduced into 5 velcro-net bags (**Figure 5.2b**). These bags are placed inside the release box machine in order to accompany the rest of the sterile flies during the ferry time and time of release (**Figure 5.2a**). When the plane comes back, the velcro-bags are transported to the quality control laboratory, where the flies must be briefly chilled to handle them and then conduct the absolute fliers and survival tests as described above.
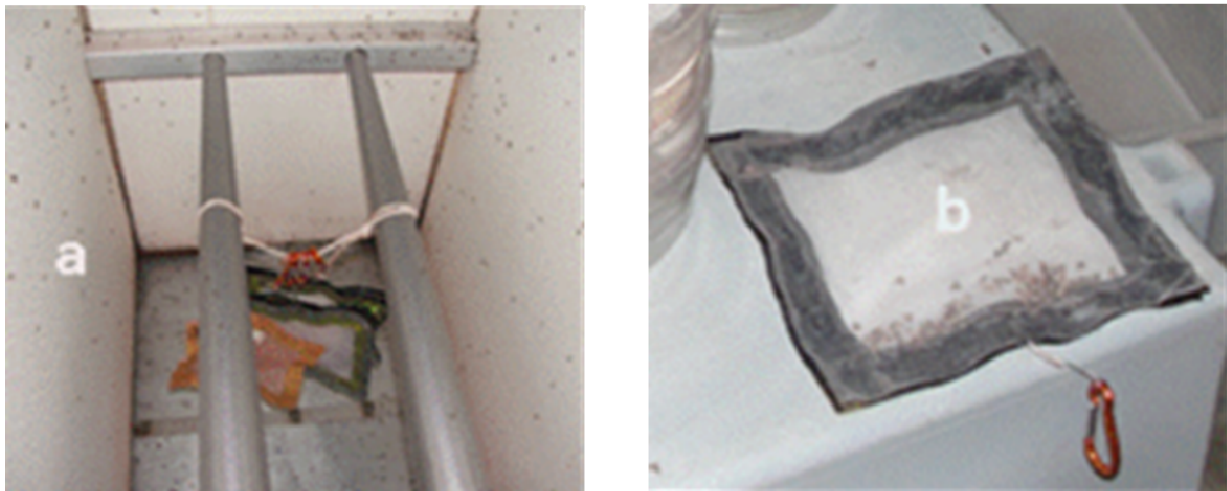
Figure 5.2: (a) Velcro bags containing chilled sterile flies and placed inside an aerial release machine; (b) Velcro-bags containing 100 chilled flies to perform the tests of absolute fliers and survival under stress.

Interpretation

Adult Fliers is determined by the weight difference between the total flies in the sample and the non-fliers that remained inside, and adjusted by the percentage of adult emergence. Percentage of adult fliers is calculated according to the formula showed in section 5.3.1 Adult Fliers.

5.4.2. Post-release survival Test

Objective

Determine survival of sterile insects after having undergone all the handling steps and their release into the field.

Discussion

The determination of adult fly survival after rearing, irradiation, shipping handling, chilling and release is a relevant test to predict how successful the SIT could potentially be if sterile insects survive well after the whole process. This test should be conducted with food and water to assess their survival potential. In addition, the test could be run in parallel with water and no food to estimate nutrient reserves of the sterile flies.

Equipment and Procedures

Five samples of 20 ml each must be taken randomly from the collected chilled flies, and carefully introduced into five Velcro bags. These bags are placed inside the release box of the ground or aerial release machine in order to accompany the rest of the sterile flies during the ferry time and time of release (**Figure 5.2a**). When the release box returns, the velcro-bags are transferred to the quality control laboratory, where the flies are released inside one 30x30x30 cm Plexiglas cage. Five samples of 100 flies are taken and placed inside a fine screened cage of similar size that can be kept in the laboratory or placed in the field. At least every 24 h the number of dead fly must be registered, although more accurate data can be obtained if recorded more frequently. The test should be extended to the time required to exceed 50% mortality. Recommended test frequency is monthly.

Interpretation

Survival is expressed as the number of accumulated hours in which 50% of flies survive. This final determination of sterile fly survival after the whole rearing and release process is relevant as a baseline to compare with field survival measures such as data coming from Release-Recapture Dispersal and Survival test and Survival in the Field test.

5.5. Relevant Literature

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6. Required periodic quality control at the mass-rearing and/or fly emergence and release facilities

6.1. Sterility Test

Objective

To ensure that flies being released into the field have the required degree of sterility.

Discussion

The biological assay of radiation-induced sterility is an essential component of quality control of the radiation procedure. It is generally carried out both at the production facility and at the fly emergence and release facility.

Uniformity of physiological age at irradiation is a critical determinant of the extent to which sterilization procedures affect the quality of flies. The optimal age at the time of irradiation is typically 24-48 hours prior to adult emergence. Irradiating pupae too young lowers the insects' overall vitality, whereas irradiation too close to emergence may result in incomplete sterility.

Equipment

- Emergence cage.
- Test cages (Plexiglas or screen) with water and food.
- Aspirator or suction pump.
- Petri dishes with lids lined with dark, absorbent substrate (e.g. black filter paper), over pieces of synthetic sponge cut to fit; moistened with water.
- Incubator or other environmentally controlled space.
- Dissecting microscope and equipment
- Fine paintbrushes.

Test Conditions

- Temperature $25 \pm 1°$ C
- Humidity $65 \pm 15\%$ RH
- Light intensity   1,500 lux
- Photoperiod   14 hours light:10 hours dark

Test Frequency

Assessment of sterility is essential if a component of the irradiation process has been significantly altered: a change in the required dose, a different irradiation source, different age of pupae at irradiation, different irradiation container. Anything that may alter the amount of radiation received by the pupae will affect the level of sterility induced in the resulting flies. Although the test is required periodically, it can be run routinely for every shipment to ensure that the radiation process has been carried out properly.

Procedure

Pupae should come from a batch destined for release in the field. A random sample of approximately 600-800 irradiated pupae should be taken ensuring that pupae from all positions in the container are represented. Another sample of approximately 600-800 non-irradiated pupae should be set apart.

Irradiated and non-irradiated flies are separated by sex within several hours of emergence. Fifty males and 50 females (or more, if feasible) are transferred using an aspirator or preferably a suction pump into each of three test cages. One cage is set up for each combination of sex and irradiation treatment:

1.) Non-irradiated male x non-irradiated female (control)
2.) Irradiated male x non-irradiated female (male sterility check)
3.) Non-irradiated male x irradiated female (female sterility check)

In the case of genetic sexing strains, the female sterility check is not needed.

Flies are provided with food and water *ad libitum* for the duration of the test. After a number of days (depending on the species, e.g. 4-5 days for *C. capitata*) the flies become sexually mature and begin to mate. When the females approach the age at which oviposition begins, they are provided with an oviposition substrate that is appropriate for the species and strain and from which eggs can be readily extracted. If the females oviposit through a cloth or screen, a section in one side of the test cage must be replaced with a screen and a Petri dish of water placed beneath it to catch the eggs (**Figure 6.1**).
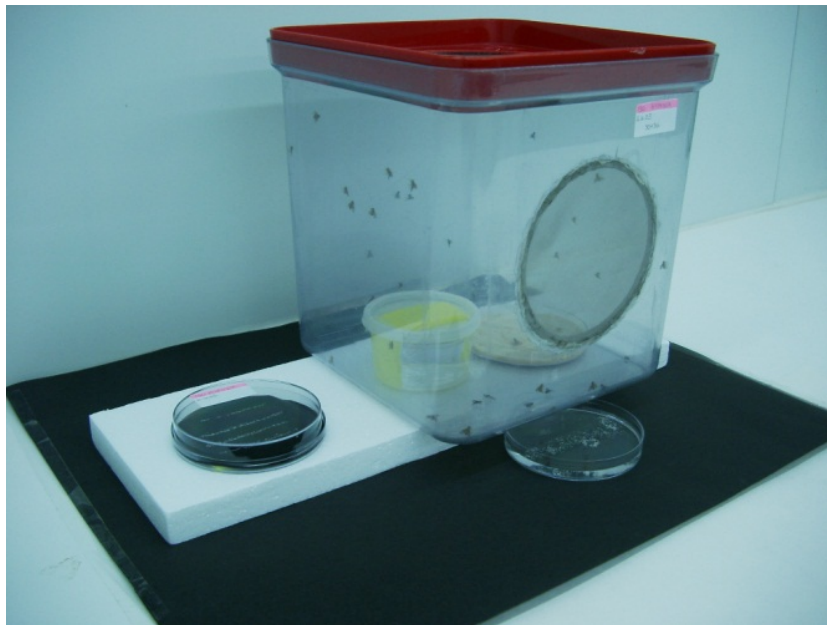
Figure 6.1: Set up for sterility tests. Cage (20 x 20 x 20 cm) has a screen in the lid for ventilation and a fine stainless steel mesh in the front face for oviposition. Insects are provided with water on a soaked sponge and adult food (sugar and yeast hydrolysate mixture). Eggs fall into water in the Petri dish below the mesh and are streaked onto wet black filter paper in a Petri dish for determination of hatch.

The first eggs produced should be discarded as not all of the females may have mated. From approximately the 5th or 6th day (for Mediterranean fruit fly) eggs should be collected daily from each of the cages and streaked onto wet filter paper in Petri dishes. A sample of a minimum of 100 good eggs (not dehydrated, stained, clear, or otherwise damaged) should be collected from each cage on five consecutive days. The covered Petri dishes are held for up to five days until hatching is complete. Numbers of hatched and unhatched eggs are then counted and registered in the appropriate form (section 9.6 Sterility Assessment Form).

For routine tests the minimum number of eggs to be collected is 500; however, for a more detailed sterility study larger numbers of eggs (>3000) are required.

Interpretation

The degree of sterility required is dependent upon the needs of the programme (suppression, eradication or preventive releases) and typically represents a trade-off between the conflicting goals of high sterility and maximum competitiveness. Any egg production by females that were irradiated at levels used for sterilization in SIT programmes indicates a problem with the irradiation process or age of pupae at irradiation. Hatch from non-irradiated male x non-irradiated female (control) crosses should be typical of what is seen in the rearing facility. The hatch in crosses involving irradiated flies needs to correspond to that requested by the end-user. Any increase in fertility indicates that irradiation procedures need to be checked.

6.2. Mating Performance Field Cage Test

Objective

To observe the mating behaviour and interactions during the time of sexual activity of sexually mature yet virgin flies from a wild population and from the laboratory strain that will be used in the operational programme in order to determine if the sexual behaviour of the flies from the mass-reared strain is similar with that of the target wild population.

Run the test once a year with 10 replicates

Biological material	Equipment	Procedures
Wild males and females Sterile males and when no genetic sexing strain is available also females	Aspirator cage	**Run the test once a year with 10 replicates** **Before the test** • Sex flies • Place males and females in separate rooms • Use sterile males from the release facility prior to/after release • For wild flies provide conditions that resemble the field • Mark the flies **During the test** • Release males • Wait 15 minutes • Release females • Survey the tree every 15 minutes for mating couples • Fill the form with required data (position and time of mating for each couple) • Check until flies separate and record separation time **After the test** • Remove the flies • Clean the cage • Wash the trees • Estimate mating performance indices **Run the test once a year with 10 replicates**

Specific Objectives

a) Assess sterile male sexual competitiveness:
To determine the ability of sterile males to compete with wild males for mating with wild females under semi-controlled field conditions.

b) Assess sexual compatibility between two fly origins:
To determine the degree of sexual compatibility between two populations/strains under semi-controlled field conditions, usually a mass-reared strain and the target wild population.

c) Increase fruit fly sexual behaviour information:
To understand failures in the sexual performance of sterile males and determine ways to improve it by increasing the knowledge of the sexual behaviour displayed by the flies under semi-controlled field conditions (e.g.: timing, location and aggregation of "calling" males, male-male interaction, courtship and female rejection, effect of male enhancers, male

periodicity of mating, female remating frequency or overall competitiveness of flies based on resulting egg hatch, known as the Fried test). Procedures of these optional evaluations are described under Ancillary Tests (Chapter 7).

Rationale

The success of the SIT relies in the introduction of sterility in the target population by the release of sterile males. The evaluation of the quality of the mass-reared males, the selection of which strain should be transferred to mass rearing, and the effect of sexual enhancers or any other supplement provided to the male before its release, can be assessed in Mating Performance Field Cage Tests. The general procedure consists of the release in a confined semi-natural environment of sexually mature yet virgin flies from a wild population and from the laboratory strain that will be used in the operational programme, in order to observe their mating behaviour and interactions during the time of sexual activity. Ideally the wild population should come from the target population of the SIT programme or from a geographically close population and the test should be run in the same environment where the flies will be released. However, under some circumstance this cannot be achieved such as when no enough number of wild flies is available or in fruit fly free areas which perform preventive releases. In those cases, the use of wildish strains (i.e. recently colonized flies with less than five generations under laboratory rearing at relaxed conditions preferably with natural fruit as larval substrate) or running the test in other areas are the best solutions. Rearing facilities may also wish to run this test to ensure the quality of the flies before shipment to the fly emergence and release facilities.

The mating performance field cage test is the best compromise between laboratory conditions and costly and impractical field observations to assess tephritid fly mating behaviour under semi-natural conditions and from there, to infer their behaviour in nature. Although procedures in mating performance field cage test aiming to answer questions that deal with the competitiveness of the sterile male and the sexual compatibility between the laboratory strain and the wild population are in general similar, the specific objectives and the way in which data is analyzed and interpreted, differ and it is important to know why and when to answer each question.

Male sexual competitiveness refers primarily to how readily wild females accept sterile males as mates in the presence of wild males. It was shown that effects of the artificial rearing system could bring about changes in the rearing colony that can reduce the acceptability of or mass-reared males to wild females most notably in shortening some sequences of the male courtship or in changing qualitatively the pattern of some sexual behaviour. For research purposes, techniques such as slow motion video recording are used to detect such changes in behaviour; however, in action programmes, the impact of such changes on the male sexual competitiveness can be assessed in a cost-effective manner by observing the ability of sterile male flies to compete with wild males for mating with wild females under field-cage conditions. During the course of an action programme, the sexual competitiveness of sterile males should be monitored periodically (i.e. once a year or preferably every 6 months). Any significant decrease in the competitiveness should lead to deeper studies and detailed evaluations of the sexual behaviour of the flies in order to decide whether it is appropriate to

change rearing procedures or to replace the strain under mass-rearing before it affects adversely the overall effectiveness of the SIT programme.

Sexual compatibility is the degree in which two groups of animals, when in contact, tend to mate randomly without regard to their group of origin rather than mating selectively with members of their own group. For tephritid SIT programmes, the sexual compatibility between the target wild population and the laboratory strain that is intended to be used for release should be measured before initiating any large-scale operation. In some tephritid species, sexual incompatibility could reveal the presence of sexually isolated populations and, to some further extent, the presence of cryptic species previously undetected. During the course of a SIT programme, the sexual compatibility should be re-assessed whenever a new laboratory strain is intended to replace the existing one.

The standard design specified here is an adaptation of the original design based on the conclusions of four groups of scientists who jointly participated in four FAO/IAEA Coordinated Research Projects (CRPs), one on Mediterranean fruit fly mating behaviour (1994-1999), one on quality assurance of mass-produced and released fruit flies (1999-2004), one on improving sterile male performance (2004-2009) and one on the resolution of cryptic species complexes (2010-2015) and from which there are available several publications and joint proceedings. As it is designed, Mating Performance Field Cage Test generates simple, reproducible, meaningful indices of male sexual competitiveness and sexual compatibility that can be used for tracking the performance of sterile flies and making comparisons between strains and other rearing and handling treatments. Although, other versions of field cage tests are available and useful in particular for research purposes, one of the main conclusions during these CRPs was that the nutritional status, sex ratio and density of flies in relation to the available canopy surface in the field cage influences test results. For this reason, efforts should be made to strictly follow the standards described thereafter in order to minimize the impact of these variables on the overall results and thus permit comparisons among different facilities and action programmes.

Source and Handling of Flies

a) Wild flies:

Wild flies should be collected from the field as infested fruit; typically, from the main host fruit available in the region. Fruit are placed on wide mesh trays or racks over a pupation substrate such as sand. The substrate is sifted every 2-3 days and pupae are collected. Pupae are then placed in a screen or Plexiglas cage used for "Emergence and Flight Ability Tests" with water and food. Food should resemble what flies obtain from nature, being one possibility an open fruit and a protein source. Recent studies are showing that several species are able to balance their nutrient intake from different food sources, so it is recommended to offer them two food sources such as an open fig plus a source of protein (such as the standard 3:1 sugar: hydrolyzed yeast). Using high ratios of sugar:protein can be also a good compromise. Within a few hours of adult emergence, select only flying adults and separate the sexes, preferably using an aspirator. Place the flies in laboratory cages (screen or Plexiglas) with a density of 30 ± 10 flies per litre volume for the case of *C. capitata* and preferably less for bigger flies such as some *Anastrepha* or *Bactrocera* spp (20 ± 5 flies per litre volume). Sexing has to be 100% effective; female cages in which even one male is

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detected cannot be used for the tests and has to be discarded and the same is recommended for male cages in which one female has been misplaced. For this reason, is better to use many small cages (i.e. 1 L) rather than a single big cage with all the flies that had been sexed. It is also recommended to sex more flies than the required to have sufficient numbers at the time of the test. Hold the flies according to the day of emergence with water and food until sexually mature. Given that age of sexual maturity may vary with species and geographic origin, preliminary tests may be required to determine the appropriate age for some still not well known species; for others there are sufficient data (**Table 6.1**). Although flies should be sexually mature, it should be considered also that flies that are presumably well past their "normal" age of mating in the field cannot be used because unmated wild females, as they age, may become more and more willing to accept less-than-optimal males as mates. During this sexually maturing time, cages may be held indoors at room temperature (around 25 °C) with a 14:10 L:D cycle; best results may be obtained by holding flies outdoors in a shaded, protected location (e.g., an insectarium) in the vicinity where tests will be run. In any case, moderate temperatures and humidity, clean water (cotton wick changed regularly) and daily light rotation of cages should be maintained to promote survival and to limit unnecessary stress.

b) Sterile flies:

Pupae should come from a mass-rearing facility and rearing procedures should follow those that are normally used for male release. Only in particular evaluations pupae coming from other instances such as from the filter colony should be used. Place several thousand irradiated dyed pupae in the devices used for "Emergence and Flight Ability Tests" as done for wild flies and follow the same procedures. Within a few hours of adult emergence, select only flying adults and separate the sexes. Assure that sorting by sex is 100% effective. Hold the flies according to the day of emergence in laboratory cages with water and the same food they are provided before release in the operational programmes. During the sexual maturation period treat the flies as they will be treated in the fly emergence and release facility. Any other aspect that takes place during the preconditioning/release stage should be considered such as the use of sexual maturation substances (e.g, methoprene), sexual enhancers (GRO or methyl eugenol), chilling before aerial releases, etc.

If the test is to be run at the fly emergence and release facilitiy, it is advisable to take the flies directly from those that are going to be released. If this is chosen, flies should be allowed to reach the age of full sexual maturation (**Table 6.1**) and in the case of bisexual strains females should be sexed soon after emergence, or at least at the age at which flies are released. If sexes are kept together and they reach sexual maturation, males will deplete their sperm load before being evaluated in the field cage.

Table 6.1: Recommended fly's minimum age (days) at which to perform field cage tests according to the fruit fly species.

Species	Wild flies	Mass-reared flies
Ceratitis capitata	7-10	4-6
Anastrepha ludens	16- 21	10-15
Anastrepha obliqua	15-17	8-10
Anastrepha fraterculus	15-20	10-15
Zeugodacus cucurbitae	30	10
Bactrocera dorsalis	25-30	10-15
Bactrocera tryoni	20	10

c) Mass-reared fertile flies:

For the case of mating compatibility evaluation, laboratory flies should be evaluated fertile to remove any impact of the sterilization procedure that may result in a reduction of heterotypic matings. Procedures to handle pupae, sex flies and feed the adults should equal to those described above. For compatibility purposes adult diet should be the same as for wild and mass-reared flies.

Holding conditions: During the time of sexual maturation sexes should be placed in a separate rooms.

Note on the type of food to provide the flies: Selection of the right food to provide the flies is crucial for the outcome of the test. Adult food has been shown to affect male sexual competitiveness in several aspects. In particular, males who ingest protein as adults have several sexual advantages over those that feed only on sugar based diets. It has been advised that facilities should provide some protein in the pre-release diet and this has been adopted in some of them. In addition, it has been proven that sterile males of some species are able to find and exploit sources of carbohydrates in nature and those fed with protein before their release remain with higher protein contents for the first few days after release compared to males that were deprived from protein in their pre-release diet. It has also been shown that protein can be detrimental and reduce longevity. Recent studies are providing evidence that it is not protein in itself but the sugar:protein ratio that is ingested what affects survival. In all, evidence is showing that protein ingestion provides sexual advantages and that the detrimental impact can be reversed adjusting the sugar: protein ratio. It is important then that the food the flies will receive before they are released in the cage resembles what they will receive really in the operational procedures. However, it may be argued that sterile males before joining the leks may forage for additional nutrients if necessary. Hence in those programmes in which no protein is added to the pre-release diet, sterile flies should receive some protein source (eg, the same food of the wild flies) one or two days before the field cage tests are performed. In those cases, it is strongly recommended that further studies are performed to evaluate the impact of adding a protein source on the competitiveness of the flies.

Equipment

- All necessary equipment to maintain the flies in the laboratory from emergence until release in the field cage: Plexiglas cages with "Flight Ability" devices, aspirators to sex the flies, 1 L containers, water and food containers, etc.
- All necessary equipment to mark the flies according to the chosen technique (see below): (1) fluorescent powder; (2) water-based paint, thin soft camel hair brushes and a meshed bag; (3) food colorants to dye the diet (i.e. those use for culinary purposes).
- Outdoor field cage, 2 m tall by 2.9 m in diameter, set up over a plant that fills a large portion of the volume of the cage (**Figure 6.2**). Ideally, the plant should be rooted in the ground, but potted plants may suffice if ground-rooted plants are unavailable and can be a local host plant of the fly species to be tested (citrus, guavas, etc.). Artificial trees may also be an option and have the advantage that flies are not exposed to plant volatiles that alter flies' behaviour. The available foliage must provide an abundant substrate for mating behaviour, but could be lightly pruned (if necessary) so that flies will be visible to the observer. An average of 20 medium-sized leaves should be available per fly released in the field cage. If adequate foliage and light are available within the cages and if no more than 150-200 flies are released per cage (regardless of the type of field cage test done), little if any mating activity should take place on the screen of the cage.

Figure 6.2: Standard walk-in field cage used for mating performance test.

- Dental cotton wicks impregnated with water to be placed in the trees as source of water for the flies.
- Plastic pill vials, scintillation vials, or similar containers of about 10-20 ml preferably clear, to collect the mating couples (depending on the amount of flies released, 50-80 per cage).
- Grease pencils and/or masking tape and pens for marking vials.
- Data recording forms with pencil.
- Hydro-thermometer, luxmeter or automatic data loggers.

Long-wave ultra-violet lamp (should flies not be marked with paint and identifiable only by the presence of fluorescent dye) or fluorescent microscope.

Procedures

Before releasing the flies in the cages, it would be necessary to mark them in order to distinguish at which strain/population they belong. Three methodologies are widely used and can be applied as convenient even in conjunction:

(1) dyeing with fluorescent powder as in operational programmes,
(2) painting the flies in the thorax with water based paint or
(3) providing the flies with diets dyed with non-toxic food colorant.

Each technique is applied at different times. In the case of fluorescent powder, dyeing occurs at the pupal stage before adult emergence with approximately 1.5 g per litre of pupae, procedures are those explained in section 3.2 Pre-irradiation Procedures.

In the case of painting the thorax, at least 24 to 48 hours before the test, flies are marked individually by applying a small dot of paint on the dorsal surface of the thorax. Immobilize flies by placing them in a bag made of mosquito net (18 mesh) and holding the mesh down gently around each fly, one at a time. Use a thin, soft camel hairbrush to apply a small drop of paint to the fly (**Figure 6.3**).

For the case of adding the colorant to the diet, it is advisable to do it since adult emergence. Non-toxic food colorant is added to the adult food in order to obtain the same food with different colours. Flies that ingest the food will have their abdomen coloured with the provided colour as illustrated in **Figure 6.4**. This marking technique has shown to be reliable for *C. capitata* and several *Anastrepha* species and reduces fly´s handling compared to the technique which involves painting the thorax.
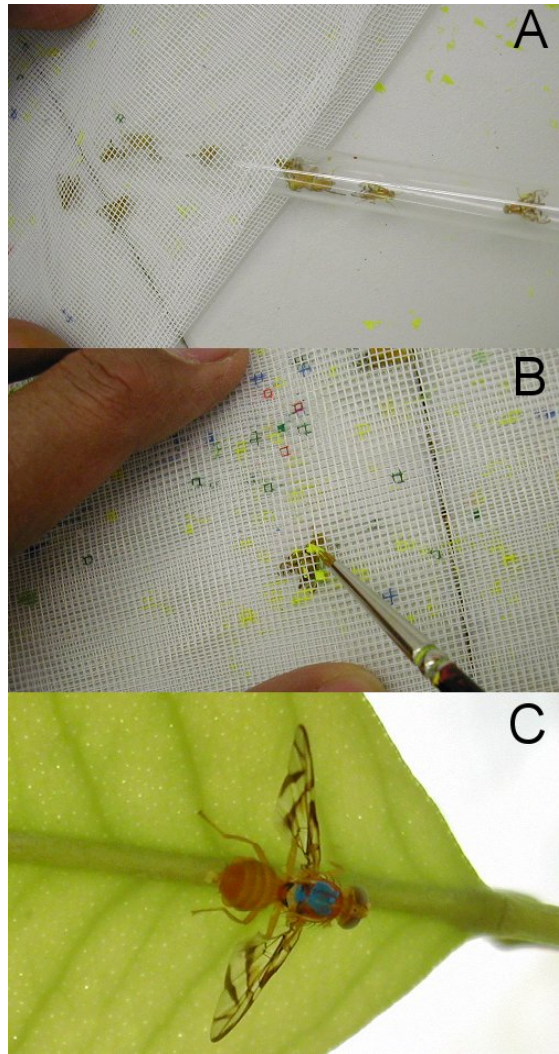
Figure 6.3: Procedure for marking tephritid adult individuals with water-based paint. (A) adults are gently blown from the aspirator into a bag; (B) the bag is stretched to immobilize the fly and a drop of paint is made on the notum; (C) the result: a marked *Anastrepha fraterculus* male calling in a field cage.

Figure 6.4: Flies marked with non-toxic food colouring.

Even though marking of flies with water-based paint or food colorants appears to have no effect on mating performance field cage test results, colours used for marking strains should be exchanged randomly among replicates and brands of paints or colorants could be evaluated ahead of time to ensure that the mark does not affect fly behaviour or survival.

Immediately after marking, flies are transferred to containers suitable for releasing them into the field cages with water and food in groups of 25-50 flies, depending on the species, per 1 litre container (**Figure 6.5**).
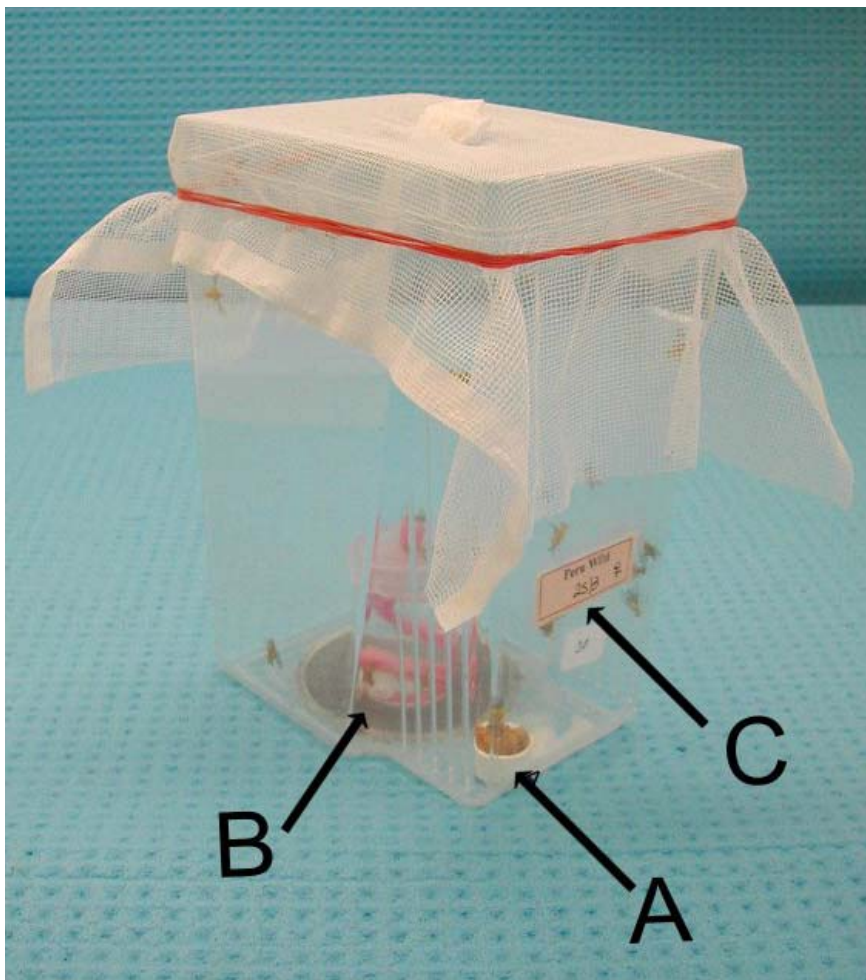
Figure 6.5: Container used to hold the flies after individual marking and before releasing into the field cage. A: food; B: water; C: label mentioning the strain, sex and number of adults.

On the day of the test, wild and sterile males are released into a screen cage and given a period of time (e.g., 15-30 min) to disperse and establish territories. The number of flies to be released depends on the species; for the case of the Mediterranean fruit fly normally 50 flies of each strain and sex are released which totals 200 flies per cage (when both males and females of the lab strain are released) or 150 flies (when only males, like for GSS, are released from the lab strain). For the case of *Anastrepha* or *Bactrocera* species the number is around 25-30 flies per sex and origin. Time of release should precede the time of peak mating activity for that species. For example, adults of *C. capitata* and *A. fraterculus* are typically released around dawn; while for *B. dorsalis* tests are run at dusk. Flies should be able to fly out of the container by themselves and should not be forced to do so by shaking the container or pushing flies out. Flies that are left inside the container, dead, deformed, or apparently

incapable of flying should be replaced so that the desired number of "normal" flies per strain per sex is maintained. After the 15-30 min period of acclimatization of the males, females are released.

After release of females, observers should screen the tree for mating couples. This can be done continuously or a census of mating couples can be done under a certain frequency. Census frequency should be around 15 minutes and can be greater only in those cases in which mating duration is longer than one hour. Capture mating couples individually in the vials, taking care to get only one pair per vial (**Figure 6.6**). Look at the pair carefully in order to confirm that copulation is taking place; males sometimes mount the female for a certain time until copulation starts. It may also happen that males sometimes mount other male resembling a mating couple. If during this procedure you lose one fly of a pair, release the other. If you have three flies, remove the extra (usually male) fly. Do not put two pairs in the same vial.

Figure 6.6: Collection of an *Anastrepha fraterculus* mating pair during mating performance field cage test.

Write on the form the type of male and female, the time when pairs were captured and the position on the tree (height, substrate, etc.). Label the vial accordingly, indicating the number assigned to the field cage in which the mating took place (if more than one cage is run on a given day), day of test and the number of the mating pair (couples should be numbered in the order they are collected in a given cage, starting from 1, for the first mating pair). This is extremely critical and attention should be paid in order not to mix the mating pairs, particularly if they will be used in any of the complementary tests or if the flies are marked with fluorescent dye. In this case strain identification will take place later under the UV lamp or the fluorescent microscope. Ensure that the vials are kept in the shade to minimize thermal or other stresses to the flies until the couple disengage. Record in the form the time mating ends.

Continue the test until the natural period of peak mating for that species under local conditions is well over. In the absence of such information, the test should cover most of the day. Once the test is over, with the aid of an aspirator remove the flies that did not mate from the cage. It is advisable to wash the tree to help removing any chemical signal left by the flies if the cage is to be used on the following or consecutive days.

Ideally, tests should be run "blind"; i.e., personnel running the test should not be told which colour of marking corresponds to wild or sterile flies.

Note on replacing flies that mate: Although it has been proposed that once a couple is removed from the cage it is desirable to release one male and one female of the given origin so the ratios of all the flies remain constant, this may be impractical in some cases and require more flies. A compromise solution is not to extend the test to situations in which more than 75% of the flies mate. Again, the decision of removing some couples from the data analysis is recommended once the data is gathered and not while running the test.

Note on mating performance field cage test with genetic sexing strains (GSS): If the mating compatibility of a thermal lethal sensitivity *tsl* GSS is to be evaluated, then use 50 females from the rearing colony stock (not thermally treated). Data from such tests may be unreliable if temperatures within the field cage are high (i.e., >27 °C) so special care should be taken if conducted at high temperatures. In case of evaluation of male sexual competitiveness of any GSS, it is not necessary to release laboratory females in the cage.

Note on addition of sexual enhancers: As stated above, the main objective of the Mating Performance Field Cage Test is to evaluate the sterile male sexual behaviour in a situation that resembles what is happening in nature after sterile fly releases. As such, sexual enhancers should be added only if the action programme uses them routinely.

Interpretation

Several indices have been developed to quantify the sexual performance of the flies, including competitiveness and compatibility. For each cage compute the number of matings in each of the four possible categories (sterile males with sterile females (SS); sterile males with wild females (SW); wild males with wild females (WW); and, wild males with sterile females (WS) or of the two categories (SW and WW) should the mating performance field cage test be run for GSS strain. Analysis of test results should involve the use of the main indices available and it is advisable to include $\chi 2$ Goodness of Fit analysis to assess statistically any significant departure from random mating. They should also be presented in graphical form as shown below for each index. For meaningful data, the basic mating performance field cage test should ideally include in total 10-15 replicates performed during 5-10 days with at least 6-10 different batches of sterile flies. Additional replicates are desired but unnecessary if variability among replicates is low.

Proportion of mating (PM): The first thing to look at is whether the flies mated during the field cage tests by estimating the proportion of mating. The PM measures the suitability of the flies and the environment for mating and is defined as:

$$PM = \frac{\text{No. of pairs collected}}{\text{No. of females released}}$$

Mean percentage of mating provides a useful indication of mating propensity. For instance, in *C. capitata*, mating propensity is considered adequate when 50% of flies from all combinations of strain and sex participate in mating. In practice, flies from some wild strains (especially the females) are more reluctant than sterile females to mate in field cages. At any rate, data from a cage should be discarded if less than 20% of both males and females from any strain participate in mating.

It may happen also that a significant number of matings take place in the screen of the cage rather than in the tree. Test replicates with more than ca. 25% of wild flies on the screen of the cage may reflect inadequate environmental conditions (such as inadequate light, lack of water, leaves, etc.) and should therefore be repeated. It is agreed that adequate environmental conditions within a field cage are reflected by wild flies remaining on the tree canopy. Should more than a small proportion of mating activity occur on the screen, cage shading and other conditions must be adjusted to correct this situation. In addition, it is recommended that couples collected away from the tree should not be included in the data analysis. This statement, however, should be taken with caution in compatibility tests given that the occurrence of leks and matings in the screen of the cage may also reflect some kind of spatial (i.e. ecological) isolation. For this reason, it is recommended that any information should be recorded during the test and evaluated carefully during data analysis.

Relative Sterility Index (RSI): The RSI is the major index to quantify male sexual competitiveness, as represented by the formula:

$$RSI = \frac{SW}{SW + WW}$$

Values of RSI can vary from 0 to 1, where 0 indicates that all of the wild females that mated in the cage mated with wild males, 1 indicates that they all mated with sterile males, and 0.5 indicates that half mated with sterile males and half with wild males and that sterile males are equally competitive with wild males (**Figure 6.7**). For *C. capitata*, a mean RSI of less than 0.20 in a cage with a 1:1 ratio of S:W is a reason for concern about the competitiveness of the sterile males.

When the released ratio differs from 1:1, then the expected RSI to compare should be adjusted. For instance, if the released ratio is 2:1, sterile to wild, then the expected RSI (where the competitiveness is equal for sterile and wild males) is 2/3 or 0.667; if it is 3:1 sterile:wild, then the expected RSI is 3/4 or 0.75, and so on. Then by comparing the observed and the expected RSI is possible to determine the actual competitiveness of the sterile males.

$$RSI = \frac{SW}{SW + WW}$$

Figure 6.7: Graphic representation of the Relative Sterility Index (RSI). The value shown (mean ± se) was obtained when comparing wild and sterile *Anastrepha ludens* flies in field cages (after Hernandez et al. 2003).

Competitiveness Index based on the RSI (CRSI): The CRSI is an alternative to have an idea of the competitiveness of the sterile male relative to that of the wild male and is represented by the formula:

$$C_{RSI} = \frac{RSI}{1 - RSI}$$

This index compares the proportion of sterile male matings to the proportion of wild male matings and in the cases in which the same number of sterile and wild males is released in the cage; the expected CRSI is 1.0 if sterile male competitiveness equals that of wild males.

Relative Isolation Index (RII): The RII is a measure of mating compatibility between two strains.

$$RII = \frac{SS \times WW}{SW \times WS}$$

Values of 1 indicates random mating between strains, which is desirable in terms of SIT application; values greater than one indicate positive assortative mating, i.e., laboratory males tend to mate only with laboratory females and vice versa (**Figure 6.8**).
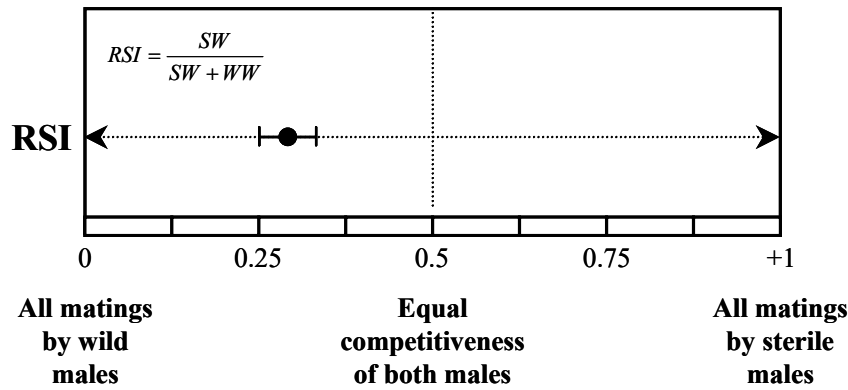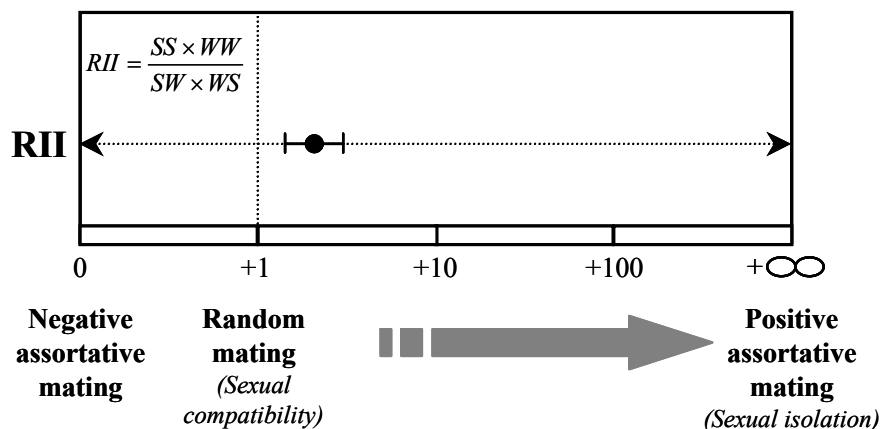

Figure 6.8: Graphic representation of the Relative Isolation Index (RII). The value shown (mean ± se) was obtained when comparing wild and sterile *Ceratitis capitata* flies in field cages.

The RII has some advantages over other indices of compatibility. For example, it is more sensitive to drops in a single type of mating, with SW being the type of greatest concern to SIT programme managers. In addition, the RII is not affected by the overall level of participation of the different types of flies, but only by whom they chose to mate. Similarly, an RII of 1 indicates random mating regardless of the S:W ratio being tested in the cage (typically, it will be 1:1). If the assumptions can be made that laboratory females accept wild and laboratory males equally and that wild and laboratory males will have equal mating propensity in the open field, then the RII is equal to the number of laboratory males it takes to equal the mating capability of one wild male in the field; the reciprocal is then the effect of mating compatibility on overall competitiveness.

But RII also has some disadvantages. First, it is undefined if SW or WS are zero. Second, when the number of matings in any one category is small, adding or subtracting a single mating in that category will cause a large change in the value of the index. Third, it can be difficult to normalize if the data are to be analysed statistically. However, the first drawback can be removed if the formula is corrected by adding one (1) to each of the four mating combinations.

$$RII = \frac{(SS+1) \times (WW+1)}{(SW+1) \times (WS+1)}$$

Values of RII larger than 1 indicate that there is some difference in mating behaviour (in a broad sense) between wild and laboratory flies that results in one or both strains tending to mate assortatively (i.e., like with like). Values of RII that trigger corrective action will probably be found to vary from species to species, and wild flies from some areas seem to consistently produce higher RII's than flies from other areas (using the same laboratory strains). For *C. capitata*, RII has typically averaged from 1.5 to 5 but in the case of sterile flies from the Hawaiian Hi-Lab strain with SIT-"resistant" *C. capitata* on Kauai, RII reached values around 30. In general, values of RII consistently larger than 3 for *C. capitata* suggest that rearing facilities should start considering alternative strains for production.

Isolation Index (ISI): The ISI is another measure of mating compatibility.

$$ISI = \frac{(SS+WW) - (SW+WS)}{SS+WW+SW+WS}$$

Its values range from -1 (complete negative assortative mating; i.e., all matings are with members of the opposite strain) through 0 (random mating) to +1 (complete positive assortative mating; total mating isolation of the two strains) (**Figure 6.9**). The main advantage of this index is that, by ranging from -1 to +1, it is easier to assess the deviation from the expected value of 0 than it is with index ranging from 0 to infinity. Compared to the RII, ISI is not as sensitive to a change in a single mating and can always be defined, whatever are the values of the 4 types of mating. In general, values of ISI consistently larger than 0.5 suggest that some positive assortative mating took place (which should be explained by analysing the values of indices that follows) and that replacing strains should be considered.

$$ISI = \frac{(SS + WW) - (SW + WS)}{SS + WW + SW + WS}$$

ISI

-1   -0.75   -0.5   -0.25   0   0.25   0.5   0.75   +1

Negative assortative mating   **Random mating** *(Sexual compatibility)*   **Positive assortative mating** *(Sexual isolation)*

$$FRPI = \frac{(SS + WS) - (SW + WW)}{SS + SW + WS + WW}$$ $$MRPI = \frac{(SS + SW) - (WS + WW)}{SS + SW + WS + WW}$$

FRPI

MRPI

-1   -0.75   -0.5   -0.25   0   0.25   0.5   0.75   +1

All matings by wild individuals   **Equal mating propensity**   **All matings by sterile individuals**
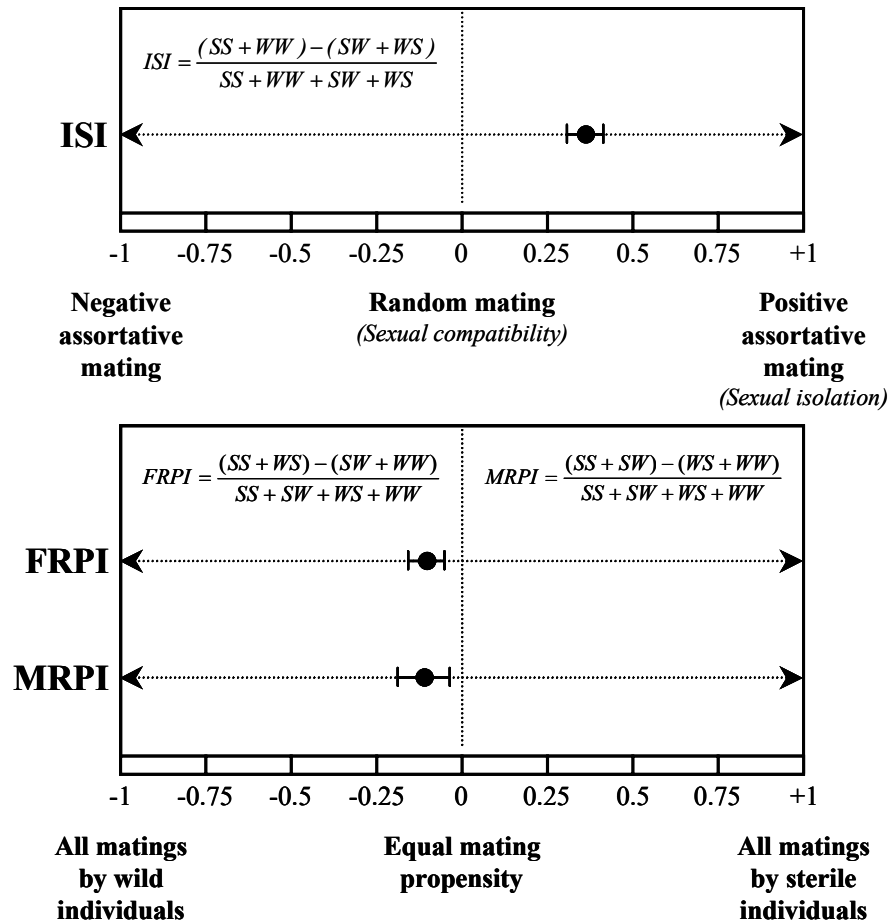
Figure 6.9: Graphic representation of the Isolation Index (ISI) and of the Male and Female Relative Performance indices (MRPI and FRPI). These indices should be considered together for a better understanding. The value shown (mean ± se) was obtained when comparing wild and sterile *Anastrepha ludens* flies in field cages (after Hernandez et al. 2003).

Male Relative Performance Index (MRPI). The MRPI is a relative measure of mating propensity of sterile males versus wild counterparts.

$$MRPI = \frac{(SS + SW) - (WS + WW)}{SS + SW + WS + WW}$$

A value of 1 indicates all matings in the cage were done by laboratory males, and -1 indicates all mating was done by wild males. Zero (0) indicates that wild and laboratory males participated equally in mating (**Figure 6.9**). In addition to RSI and the CRSI, the MRPI can be interpreted as a measure of sterile male performance. Values greater than 0 will indicate better performance of the sterile male and values bellow 0 will indicate a poorer performance with wild males achieving more matings than sterile males.

Female Relative Performance Index (FRPI): The FRPI is the counter part of the MRPI and serves as a measure of mating propensity for female flies (**Figure 6.9**).

$$FRPI = \frac{(SS + WS) - (SW + WW)}{SS + SW + WS + WW}$$

The joint analysis of ISI, MRPI and FRPI, should provide a complete and reliable picture of the sexual compatibility between strains and, should a deviation from the expected standard be encountered, the reasons why it occurred. In general, when comparing a mass-reared strain and a wild population of *C. capitata*, ISI values range between 0.1 and 0.4, FRPI values are positive values and MRPI values are close to 0. These data show that, in such a case, there is a slight tendency toward assortative mating; that is, mass-reared females are less selective in matings than are wild females and that both types of males are as competitive in mating regardless of the type of mate. Values of SW that are low compared to other possible combinations suggest that wild females tend to reject the courtship of a high percentage of laboratory males. Low values of SW and WW may indicate immaturity of wild females. Low values of both SW and WS suggest a general incompatibility between the strains.

Note for analysis based on mating performance field cage test with genetic sexing strains (GSS): In the case of GSS and tests in which mass-reared females are not released in the cage, the MRPI provides no additional information than the obtained with the RSI.

Other variables related to male sexual behaviour

Latency (Time to start copula): The time in which laboratory (either fertile or sterile) and wild flies initiate their sexual activities and mate is also a good parameter to describe the sexual behaviour, particularly mating readiness and competitiveness, and this variable has been useful to uncover differences between origins and/or deviations generated in the laboratory. Values are obtained from those recorded in the form when the couples were collected in the cage and by applying the following formula:

Latency = Copula start time - Time of onset of field cage test

Mean values are computed separately for each cage and comparisons between mating types can be analysed by means of mixed models (with male and female origin as fixed factors and batch of sterile flies, cage and test day as random factors).

Duration of mating: Length of time spent in copula can be an indication of laboratory adaptation of a strain and can be related to sperm and accessory gland fluid transfer from the male to the female. Although longer mating duration *per se* should not be considered as an indicator of male quality and the literature shows that it is not linked specifically in all species to the reproductive success of the male, it is a useful way to explore differences between strains/populations and more interestingly to uncover possible male-female interactions. In *C. capitata*, it has been noted that an exceptionally long duration of mating (>3 hours) very often resulted in no sperm being transferred to the female. However, attention should be paid if matings with sterile males are shorter compared to that with wild males. Mass-reared males tend to have reduced copulation times when compared with wild males. These reduced copulation times can be associated with increased female tendency to remate.

As for latency to mate, mating duration is estimated from the data recorded on the time in which the couple was collected and the time in which it separates. Mean values are computed for each cage and comparisons evaluated by means of mixed models (with male and female origin as fixed factors and batch of sterile flies, cage and test day as random factors).

Location of mating: The place in which the couples are collected is a good reliable indicator of where the males form their leks. In this respect recording this variable is very simple and in conjunction with that related to the time and duration of mating completes the analysis in the basic mating performance test. Data is gathered from the forms in which it has been recorded the position of the couple when it was collected. Although several aspects con be taken into account, the most relevant are related to whether the couple was found in the tree or the screen of the cage, the height in which it was collect (usually the three and the cage are divided into three parts: upper, middle and low), the part of the tree (upper side of the leaf, under side of the leaf, steam) and the quadrant respective to the cardinal axes (North, East, South, West). Frequency of matings occurring in each location can be compared by means of frequency analysis and it is suggested to run χ^2 of Homogeneity analysis.

Concluding remarks

This test if run at a periodic basis and covering all the aspects necessary to resemble field conditions will monitor sterile male sexual performance and give a good indicator of male sexual competitiveness. If mating performance of sterile males with wild females is poor or any other aspect of the sexual behaviour is not as expected, it will be necessary to conduct more detailed observations during the mating performance field cage test or to include supplementary tests in order to identify the primary cause of the observed reduction of compatibility and/or competitiveness. Procedures and interpretation of such extended observations and tests are presented in the Ancillary Tests Section. It is strongly recommended to perform any of these evaluations whenever possible. Early detections of any deviation from normal behaviour will save time and resources.

In addition, other variations of the mating performance field cage test may be conducted if deemed necessary. Examples of possible alternatives include: higher over-flooding (sterile:wild) ratios (as would be expected in the field during most SIT programmes); use of various potted host plants; use of higher male:female ratio by releasing females slowly over time (as would be expected in natural leks in the field). In this specific case, it was agreed

during the FAO/IAEA CRP on Mediterranean fruit fly mating behaviour that a relevant male to female ratio for this test should not be less than 3:1. Use of tests with alternate designs may be particularly valuable in diagnosing causes of less-than-desirable levels of sexual compatibility or male sexual competitiveness. However, for the sake of reliability, all field cage tests of any type should always include wild flies as control and they should not replace the standard protocol since it impedes comparisons of the results among facilities or fly emergence and release facilities.

6.3. Release-Recapture Test of Dispersal and Survival

Objective

Estimate the ability of released sterile insects to survive and disperse in the field.

Rationale

The ability of sterile flies to survive in the field and to move from the point of release to feeding, mating, and resting sites is obviously critical to the success of SIT programmes. Release-recapture tests can be used to evaluate both the survival and dispersal of the flies. The test consists in releasing flies in a releasing point and establishing a trap grid around this point. Traps are serviced at regular intervals to score the number of captured flies. Data from this test are useful for making comparisons between different strains, flies from different facilities and/or flies that receive different rearing procedures or pre-release treatments.

Aside from assessing fly quality, an important function for this test is to determine if the release protocol being used in the operational programme provides the adequate coverage throughout the programme area both in terms of time and space. Data from this test can be used as a basis for adjusting operational procedures, such as the time interval between two releases in any given area or the distance between release points (or flight lines).

During ongoing SIT programmes, the test should be run in an area where the climate and habitat are similar to that of the programme release zoneAs an alternative, the test can be run within the programme zone if flies for the test are marked with a separate colour. This tactic, however, requires the tester to deal with the large numbers of programme flies that will end up in the traps.

Source and Handling of Flies

Sterile flies should receive the same treatment as those from the regular releases in the action programme. This includes food regime, holding conditions and any pre-release treatment (GRO, methoprene, etc). For this reason, it is recommended to recover the flies from the releasing machine (as in section 6.4.1 Absolute fliers) or to use the flies that are ready to be released. Procedures to hold the flies before release should resemble that of the fly emergence and release facility.

Equipment

- Attractant-baited traps, appropriate for the species being tested (see below).
- Dissecting microscope.
- Long-wave ultra-violet lamp.

- Containers to hold the flies in the same way as in the fly emergence and release facility.
- Screen cages, small PVC pipe framed cages as in the Survival in Field Cages Test (in Ancillary Tests, see Figure 8.3).
- Equipment to assess fly emergence (2.xx).
- Miscellaneous cups, forceps, or other supplies as necessary.

Procedures

Sterile dye-marked insects are shipped to the release facility and handled according to the standard releasing protocol. This includes placing the pupae in the same containers, and feeding and handling the flies as if they were to be released in the target area.

a) Release:

Flies are released weekly at a single point at the centre of an array of trap sites. If possible, dye colours should be rotated weekly between at least two colours. This allows following individual releases for more than one week. Flies should be released at the normal age at which the flies would be released for the control programme. If flies from different treatments (e.g., strains, facilities and/or rearing or handling regimes) are being tested, they should all be released from the same point at the same time but with different colours per treatment. Because low proportions of the flies are typically captured, large numbers (approximately 10,000 per treatment) must be released in order to obtain meaningful data. Number of adults released are estimated based on fly emergence and flight ability tests (see chapter 2.2.2) which should be conducted in parallel

b) Trapping:

The traps used for this test should incorporate the best commonly available attractant for males of the species being tested. These include trimedlure for *C. capitata* and methyl eugenol or cue lure for the appropriate species of *Bactrocera* spp (see detailed information on traps and attractants in FAO/IAEA trapping guidelines). Traps for use with parapheromone lures include Jackson, IPMT, McPhail, Tephri traps, or any of various bucket-type traps that contain a killing agent such as naled (dibrom) or DDVP. For *Anastrepha* spp., McPhail traps baited with 5 Torula yeast pellets each are still considered the standard. Both types of attractants have the disadvantage of being attractive to the flies at certain ages and/or fly condition. For this reason, some dispersal studies have used sticky traps, which are less efficient but unbiased in the type of fly they will capture. For strong attractants care should be taken to avoid trapping a high number of flies thereby interfering with sterile flies' survival and dispersal. This could be ameliorated by reducing the time the trap is left in the field or by reducing the amount of male lure in the trap.

Traps are hung at the trap sites 1, 3, 5, 7, 9 and 11 days after each release, and, in each case, are removed after 24 h. Flies that are captured are returned to the laboratory and examined for presence of dye.

c) Plot layout:

Dictating a specific layout for traps in the field is impractical due to variation in local conditions, differences among species, etc. However, whenever possible, the following guidelines should be considered:

- Traps should be placed at a series of concentric circles. This is generally considered better than a grid layout for measuring dispersal. Care should be taken to maintain the density of traps with increasing distance from the release point.
- Traps should be placed inside the foliage of a known host tree, at about 2/3 of the maximum height of the tree canopy. Special attention should be given to remove leaves or branches in order to maintain freely accessible the entrance(s) of the traps.
- Distance between traps may vary depending on species, trap type, local topography, and available host plants. However, the plot should ideally be at least as wide as the distance between adjacent release sites (or flight lines) in the operational programme.

d) Control cages:

Cages of flies should be held within the plot to provide baseline mortality data for evaluating survival. Size of the cages can vary but it is advisable to use them as bigger as possible (as those in the Field Cage Survival Test or the ones for the Mating Performance Field Cage Test). It is important that inside the cages the flies received the same conditions as they will find in the field. For this reason, it is appropriate to include potted plants of the same species present in the releasing orchard which has been exposed in the field. This would ensure that plants have bird droppings, dew and other elements on which sterile males feed on. If smaller cages are used, then they can consist of a frame with a fine cloth containing a branch from one of trees in the orchard which will serve both for shelter and feeding. In any case, it is important to exclude crawling predators such as spiders and ants to a sufficient degree. Vegetation touching the cages must be minimized and Tanglefoot or a similar sticky substance can be applied in those points that might provide access routes for ants and spiders. A minimum of 100 flies for each combination of strain and sex should be placed in the cages (separate cages for each strain) at the time of release and are then checked for mortality whenever traps are checked. Medium size and big cages have the disadvantage that dead flies will surely be removed by ants. In this case, instead of dead flies, the observer should count number of flies that are alive for what is recommended to use a manual aspirator as an aid, and while counting place the flies in a small container. After counting, the flies are released back into the cage. As an alternative, several cages can be used and each is revised at a given time according to the time schedule to check the traps (as in the test proposed by Gomez-Cendra et al. 2007). If small cages are used, setting up 3 cages per strain is recommended. If desired, additional cages of flies may be held indoors under controlled conditions as a further check.

Interpretation

a) Survival:

Direct comparisons of numbers of capture among the different days after the release provide estimates of survival for these periods of time out in the field. This can be done visually by plotting the proportion of captured males in each trapping date. Because capture on any given day is affected by trapping conditions on that day (i.e., weather), replication is necessary to achieve reasonably reliable estimates of survival. A more precise indication of weekly survival can be obtained by comparing catch, on the day following a release, of flies that were released 1, 3 and 5 days earlier. Both of these methods will produce slight underestimates of survival unless mathematical formulas are used that compensate for the flies that are removed

from the population by the traps. In addition, the time during which the traps are left in the field can also affect the results and sometimes is advisable to reduce it to several hours instead of a whole day. High mortality in the control cages would be indicative of either unusually harsh conditions or poor viability in the released flies.

Note: Flies from some strains either are slow to develop or, for some other reason, tend to be captured in low numbers on the day after being released. Use of the above techniques, if not adjusted, will give inflated estimates of survival for such strains. For new strains, tests should ideally be conducted to estimate the relationship between age and responsiveness to attractants. For example, flies that were held for 2, 4, 7 and 10 days after adult emergence could be marked separate colours, released simultaneously into the field, and trapped the following day (only).

b) Dispersal:

While many mathematical models have been developed to describe movement of insects in the field, the simplest index for comparison among trap days, strains, etc., for these tests is mean distance ($\overline{d}$) from release site to trap:

$$\overline{d} = \frac{\sum_{i=1}^{n} x_i C_i}{\sum_{i=1}^{n} C_i}$$

where C_i is the number of flies captured in trap i, and x_i is the distance between trap i and the release point.

Given that dispersal can be influenced by host fruit availability, running the test during non-fruiting season will provide a better estimate of the capacity of sterile flies to disperse, while carrying it out during the fruiting season will provide an indication of the reduced dispersal associated with host presence which could be used to adjust release spacing.

6.4. Fly Irritability Test

Objective

Estimate the ability of mass-reared sterile insects to respond to external stimuli associated to the presence of predators (capacity to evade predators) and/or other detrimental factors.

Rationale

The ability of sterile flies to survive in the field is affected both by abiotic and biotic factors. Mass-rearing exerts selective pressures that may lead to less reactive flies. Under crowded conditions, highly nervous or irritable flies have a reduced survival. On the contrary, less irritable flies tend to have higher survival and higher mating success. In nature this irritability or capacity to respond to external stimuli may be associated to the capacity to evade predators.

Fruit flies are exposed to high predation rates in the field. It involves vertebrates and arthropods and occurs at most stages of development including the adult stage. Male pheromone is used as an orientation signal by some wasps that arrive at Mediterranean fruit

fly leks and predate over calling males. Male responsiveness, (i.e. its capacity to respond to movements in their immediate surroundings), is correlated with its capacity to evade from predation. Such ability to respond to stimuli associated with predator's presence is of relevance at the time of assessing sterile male quality.

Previous tests were conceived to evaluate male capacity to respond to external stimuli. The startle test was developed originally by Schroeder et al. (1973) and later modified by Böller et al. (1981) to determine the capacity response to light. This test was designed to be conducted under laboratory conditions and the flies are confined to small containers. Posteriorly, Hendrichs et al. (2007) developed a method to measure predation evasion under field cage conditions. The test can be done either with true predators or with the aid of an aspirator that simulates predation. More recently, Rao et al. (2014) have designed a laboratory test uses true predators.

The test presented here is an adaptation of that of Hendrichs et al. (2007). It provides a simple way to determine the ability of flies to escape from a predation event simulated by trying to capture flies released in a field cage with the aid of a manual or mechanical aspirator. It can be applied to measure strain deterioration, to compare among different strains and between laboratory and wild flies.

Source and Handling of Flies

a) Wild flies:
Wild flies are collected from the field following the procedures described in section 6.2 Mating Performance Field Cage Test. Before adult emergence, pupae should be painted with the fluorescent dye or adults should be provided dyed food after emergence and before the test.

b) Sterile flies:
Sterile pupae should come from a mass-rearing facility and rearing procedures should follow those that are normally used for male release. Only in particular evaluations pupae coming from other instances such as from the filter colony should be used. Handling procedures should follow those explained in section 6.2 Mating Performance Field Cage Test. Pupae should be painted with the fluorescent dye or adults should be provided dyed food using a different colour of that of the wild flies. Flies may come directly from the release facility at the time of their release into the field.

c) Mass-reared fertile flies:
If it is considered that irradiation might be affecting reaction capacity, then it would be desirable to compare sterile versus fertile laboratory flies. In this case, pupae should come from the same batch as those that have been subjected to irradiation. Procedures to handle them should equal that of sterile flies.

Equipment

- All necessary equipment to maintain the flies in the laboratory from emergence until their release in the field cage (see details on material in section 6.2 Mating Performance Field Cage Test).
- All necessary equipment to mark the flies (see details on material in section 6.2 Mating Performance Field Cage Test).
- Outdoor field cage, 2 m tall by 2.9 m in diameter, set up over a plant that fills a large portion of the volume of the cage.
- Battery-power mechanical aspirator (suction pump) adapted with a glass tube at the end of the aspirator to collect the flies. The tip of the glass tube, from where the flies are aspirated, should have a 5 to 10 mm diameter opening bent at an angle of 70-75 degrees to facilitate reaching the flies. With this mechanical aspirator it is assured that the simulated predation events are done in a uniform manner wherever the test is conducted.
- Plastic container to release the captured flies.
- Dissecting microscope with fluorescent light or long-wave ultra-violet lamp to identify flies if marked with the fluorescent powder.

Procedures

Keep the flies under the same conditions and dietary regime until their release in the field cage after achieving sexual maturation. On the day of the test, release 150 flies of each origin and/or sex to be evaluated in the cage. Allow 15 minutes for fly acclimatization. Capture 20 flies with the aid of the aspirator at intervals of 1 - 2 minutes per capture. Avoid attempting to remove flies that are in the cage screen given that they have more chances to detect the approximation of the observer. Leave the cage and identify the origin of the captured flies under the UV light (ideally the observer should not know the origin of the flies at the moment of capture). Repeat the procedure at least at three different times of the day to cover all the activity hours of the flies. Perform a total of 10-15 replicates trying to cover 5-10 days with at least 6-10 different batches of sterile flies. If capture probability is to be associated with any particular activity (pheromone calling, resting, feeding, etc.), special care should be allocated to seek for flies involved in this particular activity in each set of capture attempts. If true predators are used instead of the aspirator, the test should be performed as follows: after fly release a certain time is given to allow the flies to settle. Then release the desired number of predators and leave them in the cage for a certain period of time. After this time, the observer enters into the cage and removes all the live flies. Flies are taken to the laboratory and identified under the UV light. Estimate the proportion of flies captured of each origin and perform frequency statistical analysis such as a proportion test or a χ^2 of Goodness of Fit.

Interpretation

Proportion of flies captured during a "blind" sampling effort is a good reflect of the capacity of sterile flies to evade predation relative to that of wild flies. This approximation requires however, that at least two different set of flies are released in the same cage. In cases in which no relative measure is sought, then the test should be modified to record the time required to collect each fly type (i.e. how easy or difficult is to capture them).

6.5. Relevant Literature

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7. Anciliary tests

7.1. Activity Test

Objective

To assess activity levels of sterile males compared with wild flies.

Discussion

As stated in the Fly Irritability Tests (section 6.4), mass-reared flies often exhibit decreased pattern of activity, fly less and respond more slowly to adverse stimuli, which results in an increased vulnerability to predators. This reduction in activity can be quantified under laboratory conditions with the aid of video recordings. The procedures presented here are those reported by Weldon et al. (2010), where the activity pattern of the Queensland fruit fly, *B. tryoni*, from a mass-reared colony was compared to that of wild flies.

Source and Handling of Flies

Wild flies and sterile laboratory males: As for the Mating Performance Field Cage test. However, in this case, flies can be evaluated before and/or after attaining sexual maturity.

Equipment

- All the required equipment to obtain adult flies.
- Recording cages: Transparent Plexiglas cages of approximately 1-2 L to hold flies.
- Digital video camera.
- Computer with specific software to analyse recordings (e.g., JWatcher, Etholog).
- Artificial lighting.

Procedures

Procedures can vary between tests involving immature or mature flies and/or after single-sex or mixed sex treatments. The latter option may be used to determine whether differences in activity result from the presence of individuals of the opposite sex. When flies are at the appropriate age, place 3-5 flies of one sex (in the case where only one sex is released) and 3-4 flies of one sex and one individual of the opposite sex in the recording cages. Allow five to ten minutes for acclimatization and start recording the activity of the flies for approximately ten minutes ensuring that all the flies are visible for the entire recording period. Make recordings for several groups (at least 5) during early morning, midday and late afternoon and dusk.

Interpretation

Observe the recordings in real time and choose one focal individual within the group to estimate the time spent in each of the following activities: flying, grooming, walking, interacting with others (fighting, courting) or inactive. Analyse by means of multivariate analysis of the variance (MANOVA). Differences in the activity patterns between wild and mass-reared flies may indicate that the mass-rearing environment is selecting for less active flies because highly active flies are more prone to incur physical damage (collisions) than

less active flies or spend much energy in fights or agonistic interactions that reduce their survival. In addition, under mass-rearing, moving long distances during food foraging is not necessary. Identifying ways to reduce this selection force may delay the need to replace the strains. There is evidence that this can be achieved in the medfly by providing the flies with extra surface within the cage for perching and displaying courtship.

7.2. Detailed Lekking and Courtship Behaviour Observations

Objective

To complement the observations on male sexual behaviour and female response in order to detect differences between wild and sterile flies. In particular it aims at observing in detail the timing, location and aggregation of "calling" males and male-female interactions.

Discussion

Males of lekking species display laborate courtship, which involve the emission of different signals that are scrutinized by the females in order to choose their mates. It is possible to obtain more detailed information during the Mating Performance Field Cage Tests (6.2) by releasing a lower number of flies, preferably individually labelled, and observing their behaviour during the entire period of the test. The procedures used to make these observations are flexible and will depend on the particular characteristics of the species in question. Here we provide some guidance.

Source and Handling of Flies

Wild flies and sterile laboratory males: As for the Mating Performance Field Cage Test (6.2). However, in this case, it is recommended to label each fly in order to identify and record its behaviour. This can be achieved by using different colours (if few flies are being released in the cage) or by gluing a small letter (font size # 3) on the thorax (**Figure 7.1**). Females may also be marked depending on the particular objective of this test.

Figure 7.1: Couple of *Ceratitis capitata* where the female is labeled with a small letter and the male is painted in the thorax.

Equipment

- All the required equipment for the Mating Performance Field Cage Test (6.2), including standard round outdoor field cages with host trees.

- Material to label the flies individually: waterbased paint of different colours or small letters printed in paper of different colours will allow the release of more than 50 individually labelled flies within one cage.

Procedures

To determine the extent and timing of the males' participation in pre-mating behaviours, a census may be taken at regular intervals (e.g., every 10-15 min or half hour). During the census, the number, location, and identity of each male that is "calling" (i.e. releasing pheromone) within the cage or that is involved in courtship with a female is recorded. Calling males are typically (but not always) on the underside of leaves. In the medfly (**Figure 7.2**) and in some *Anastrepha* species, they can be identified by the presence of what appears to be a drop of liquid on the tip of the abdomen (in reality, a sac is extended from the anus) as well as by inflated pouches on the pleura. Calling males may intermittently vibrate or "fan" their wings while standing in place; during fanning, in some cases the anal sac is partially retracted and held under the abdomen, and thus it is more difficult to observe

Figure 7.2: *Ceratitis capitata* male calling, can be identified by the presence of what appears to be a drop of liquid on the tip of the abdomen (in reality, a sac is extended from the anus) as well as by inflate pouches on the pleura.

For each individual male fly, the observer can assess the number of mating attempts that are rejected by the females and the number of times they are engaged in agonistic interactions with other males by performing observations of focal individuals. Mating couples can be left inside the field cage given that the individual identification allows recording mating during the test without the need to have them confined in a small vial. The observation period ends once the period of sexual activity for the species has finished.

Interpretation

Calling, or releasing pheromone to attract female flies, is an early and critical step in a male's effort to secure a mate. In a field-cage situation, numbers of sterile males observed calling would typically be as high as or even higher than the number of wild males calling.

The incidence of calling is a component of mating propensity, and a low incidence of calling by sterile males could be indicative of low fly quality or vitality. Sterile males could also exhibit a low level of participation in mating despite a relatively high incidence of calling. That scenario could occur if (1) sterile males were ineffective at attracting unmated females into their immediate vicinity (possibly owing to changes in pheromone composition resulting from mass-rearing) and/or (2) females that were attracted to sterile males seldom copulated with them.

Periodicity of calling can be evaluated by comparing the proportion of sterile and wild males that are calling at different times of the day. If it is found that sterile males are actively calling but are rejected by approaching females, then it is desirable to follow this observation with the "Pheromone Compatibility Test", described in section 7.5.

In addition, these data can also be used to check diel periodicity of sexual behaviour among sterile males in comparison with wild males and to determine whether sterile and wild males call from the same locations. Moreover, detailed observational studies permit estimating the rate at which females accept sterile or wild males by quantifying the number of female visits to the different types of calling and courting males and recording rejection or acceptance of males by females. This information is relevant if extreme incompatibility values or reduced competitiveness are detected and may lead to decisions to modify rearing conditions or replace the strain to restore competitiveness.

7.3. Pheromone Calling Test

Objective

To determine if the pheromone produced by males that were mass-reared for sterilization and release is attractive to wild females.

Discussion

Frequently, colonies of fruit flies are cultured in the rearing facilities for many generations without any influx of new genes. Mating occurs under extremely crowded conditions, and there is some question whether pheromone is used in the courtship process. Under mass-rearing, there is the possibility that pheromone production may be altered (refs - Phil to find). There is also the possibility that the ratio of the components of the pheromone may change (refs - Heath). If any of these situations occur, this could affect how wild females react to sterile males in the mating arena. The following describes a simple test that can be included while the Mating Performance Field Cage Test (Section 6.2) is being conducted.

Source and Handling of Flies

Wild flies and sterile males:
As for the Mating Performance Field Cage test (section 6.2); however, in this case it is extremely important that wild flies are matured in a distinct room than sterile flies to avoid any contact of volatiles between flies of different origins. Wild females should be held in a separate room.

Equipment

- All the required equipment for the Mating Performance Field Cage Test (Section 6.2), including standard round outdoor field cages with host trees.
- Nine small-screened cages (e.g., 10 x 5 x 5 cm) with a cord and hooks on ends to suspend cages inside each of the standard field cages.

Procedures

Place 5 sterile males in each of 3 small cages, and 5 wild males in each of 3 small cages. Suspend the small cages from branches in the shade of the tree(s) at an equal distance from each other. Alternate placement of the cages containing sterile and wild males. Release 100 sexually mature virgin wild females inside the field cage. After 10 minutes, record the number of female flies perched on or within 10 cm of each cage and the number of males calling in each small cage. Then, gently blow the female flies from the small cages and rotate the small cages. Repeat the test every 10 minutes for at least 4 periods during the peak calling time so that each small cage has occupied each position in the tree cage. Divide the number of females attracted to each treatment (male type) by the number of males calling in each treatment and determine the mean and the standard error. This test can be extended to re-test cages of the same males over sequential days to assess their capacity to attract females over longer time periods.

Interpretation

Based on experience, the sterile males are expected to call more than the wild males. If they produce similar amounts of pheromone as the wild males, one would therefore expect more wild females on cages containing sterile males. If there are substantially more females per calling male resting on and near the wild male cages than on the sterile male cages, there may be some concern either that the sterile males are not producing enough pheromone or that the pheromone composition has changed. This could conceivably affect the compatibility of the sterile male and the wild female in the field.

7.4. Female Propensity to Remate Test

Objective

To compare the renewal of female receptivity between females mated with sterile or wild males.

Discussion

To compare = female receptivity after mating with sterile or wild males.

Discussion

Remating in wild tephritid females is more common in nature than previously believed. Remating studies have shown that, within the family, the relative impact of the main factors that modulate renewal of female receptivity (i.e. amount of sperm stored and accessory gland proteins) depend on the species and can be altered by the process of mass-rearing and with irradiation. Sterile males are, in general, less able than wild males to suppress remating in wild females and this effect may be increased in males that have been colonized for a longer time. Consequently, higher incidences of remating among females that mated with sterile rather than wild males may indicate potential problems with male competitiveness.

Source and Handling of Flies

As for the Mating Performance Field Cage Test. Additional males will be required to evaluate female receptivity after the first mating. If wild males are scarce, mass-reared fertile males can be used at this point. This will ensure all males are of the same age and nutritional status.

Equipment

- All the necessary equipment as for the Mating Performance Field Cage Test (Section 6.2).
- Laboratory mating cages.
- Dissecting microscope.
- Dissecting forceps.
- Glass slides and cover-slides to place dissected spermathecae.
- Microscope to determine sperm transfer.

Procedures

The test is ideally conducted as a continuation of the Mating Performance Field Cage Test (section 7.2). Each female is identified after the first mating and on subsequent days, they are offered new virgin males, and remating is recorded. It is recommended then that this part is held under laboratory conditions to avoid dependency on weather conditions. This suggestion is valid generally, but a detailed protocol should be adapted to each species. Here again, care should be taken when collecting mating pairs during the first day to ensure that they are not disturbed to the point where they uncouple prematurely.

Once couples disengage from the first mating, females are removed from the vial and returned to the laboratory to be placed in a container with water and food either single (1 Lor with other females that have mated with males of the same type (12 - 20 L). Special care should be taken not to mix females that mated with males of different type. On the next day or after a

certain interval according to the mean time of the refractory period for this species, if known, the female is offered two sexually mature virgin wild males (or mass-reared males if wild males are not available) per female during the time of sexual activity to assess receptivity. Females mated with sterile males and females mated with wild males should be evaluated under the same conditions in order to make comparisons. Remating rate and refractory period are the variables to be analyzed. New virgin males should be offered at regular intervals (i.e., every day or every two days) for a period of at least twice the time of the mean refractory period (if known for the given fruit fly species). If possible, an oviposition substrate can be provided to the females and the number of eggs laid recorded to be used as a covariate in the analysis. If female receptivity is very sensitive to fly density, then it is recommended to run the test under relaxed conditions to allow the females to reject the males.

If desired, a set of females of each type can be used to determine incidence of sperm transfer and number of sperm stored. This is done by dissecting the sperm storage organs (depending on the species both the spermathecae and the ventral receptacle or just the spermathecae) and placing them in a cover slide with a drop of saline or water with detergent. With the aid of an entomological pin, the sperm storage organs are broken apart and the material is stirred to allow sperm to disperse. Then, the slide is covered with a coverslip, and the number of sperm present in a certain number of fields under a microscope is recorded. The number of fields will depend on the magnification used, and it is recommended to consider about 10% of the total area of the coverslip (for further details see Taylor *et al*. 2000; Perez-Staples and Aluja 2006; Abraham *at al*. 2011 and references therein).

Interpretation

For meaningful data, 25 females of each category should be analyzed. Remating rate of females first mated to different male types can be compared by means of χ^2-tests. More precise analysis can be performed using multiple logistic regression or MANOVA when appropriate. First mating duration, origin of the male and other variables of interest (e.g., nutritional status, size, exposure to pre-release supplements, etc.) should be considered as the main factors. However, this requires a deeper analysis that can be left for research purposes. In all, this test provides an insight of the quality of the male with whom the female mated for the first time. If a female mated for the first time with a sterile male has a higher tendency to mate and/or a shorter refractory period than a female mated with a wild male, then it can be concluded that the sterile male is less capable of inhibiting female receptivity. In other words, higher incidences of remating among females that were mated to sterile vs. wild males suggest that sterile males may not be transmitting sufficient sperm or accessory gland fluid to the females or both.

7.5. Cage Fried Test

Objective

To provide an estimate of the overall mating competitiveness of sterile male fruit flies.

Discussion

The index of mating competitiveness (C) is the degree of sterility produced by sterile males in the wild population and is measured as a reduction in egg hatch. The mating competitiveness of sterile males will influence the number of sterile males required to be released to induce an adequate degree of sterility into the wild population. In mass-rearing, there are many factors that can change the mating competitiveness of an insect. Fried (1971) considered that final expression of mating competitiveness could be measured in the egg sterility induced in the population. He presented a model for determining mating competitiveness using different ratios of sterile to wild males. This was adapted into a test for mating competitiveness and has subsequently been called the Fried Test. The Fried test has been conducted in lab/field cages (and open field experiments) for many years in some facilities and can be included as a follow up of the mating performance test. It basically consists in comparing the percentage of egg hatch obtained in cages where sterile males compete with wild males from that obtained in cages where only wild flies are released under field conditions. The test is not widely used given the difficulty to obtain eggs from the cages; for this reason, in this new version of the manual we included a variation in which females are returned to the laboratory after allowing them to freely mate with any male type for a few days and eggs are collected in the laboratory. Although this test is considered as an Ancillary Test, it is strongly recommended to conduct it in conjunction with the Mating Performance Field Cage Test (section 6.2) whenever possible.

Source and Handling of Flies

a) Wild flies and sterile males:
As for the mating performance field cage test (section 6.2), but it is not necessary to mark (paint) the flies if the test is to be run separately from this test.

b) Fertile mass-reared females:
Place several thousand non-irradiated, unmarked pupae in a cup in a screen or Plexiglas cage and separate sexes within a few hours of emergence. Then, hold the flies until sexually mature (**Table 7.1**) in laboratory cages (screen or Plexiglas) containing an appropriate source of food and moisture (e.g., water and a mixture of protein:sucrose).

Equipment

- Outdoor field cage containing a host tree, as for the mating performance test.
- Oviposition substrate, agar balls wrapped with parafilm or ripe host fruit (free of tephritid eggs).
- Laboratory oviposition cages.
- Dissecting microscope.
- Dissecting forceps.

- Petri dishes and sponge cloths to incubate the eggs for embryonic development and egg hatch assessment.

Procedures

a) Competitiveness field cage test:

Before the field cage test (at least the day before), flies are transferred to containers suitable for releasing them into field cages in groups of 20 or 40 flies per container (flies are provided with food, moisture, and ventilation in the containers). On the day of the test, release wild males and sterile males with wild females into the field cage. Adjust number of flies as well as sterile:wild ratio according to the species as in the Mating Performance Field Cage test. Add food and water to the field cage for the duration of the test. Release the flies before the time of sexual activities and observe for male calling and the initiation of mating. Leave the cage and allow flies to mate freely. During the first day of the test, add 16 oviposition substrates to the field cage, by hanging them in the fruit tree on eight equidistant compass points at 2 different levels in the tree.

b) Control field cage:

In another cage, release wild sexually mature males and wild sexually mature females also with food and water. Confirm the occurrence of mating and leave flies to mate freely as in the competitiveness cage. The oviposition substrates are added to the cage at the same time as above.

c) Sterility test cage:

Release sexually mature sterile males and sexually mature fertile laboratory females into an oviposition cage with food and water. The oviposition substrates are added to the cage at the same time as for the field cage. Let the flies mate freely and add oviposition substrates as well. This cage can be placed in the laboratory if necessary and the purpose is to determine the sterility achieved with the irradiation (sterility test). Cages with fertile laboratory males and females ensures that the absence of egg hatch is the result of the irradiation process.

The test is run for 4 days. At 48 hours after placing the flies into the respective field cages, infested agar balls are replaced with a new set of balls. The infested balls are dissected after removal. Eggs from the balls are placed onto moistened black filter paper in a 9-cm Petri dish. The eggs are incubated at 25°C, and after 5 days, the percentage egg hatch is evaluated. For meaningful data, at least three replicates of this test are required involving three batches of sterile males. If few eggs are recovered from the field cages, remove the females from the cage and transfer them to the laboratory to provide better conditions (i.e. higher temperature or light) to enhance oviposition behaviour. For meaningful data recover at least 25 females from each field cage and do not place males in the oviposition cages in the laboratory. This procedure should be done at least 3 days after fly release in the field cages. Once in the laboratory, provide the flies with sufficient food and water and the oviposition substrate. After 48 h remove the agar balls, collect and incubate the eggs as already described.

Interpretation

The Fried competitiveness value (C) is computed using the following formula:

$$C = \frac{W}{S} \, x \, \frac{Hw - Hc}{Hc - Hs}$$

In this formula, (W) is the number of wild males in the competitiveness cage, (S) is the number of sterile males in the competitiveness cage, (Hw) is the egg hatch from wild females in the control cage, (Hc) is egg hatch from wild females in the competitiveness cage and (Hs) is the egg hatch from lab females in the sterility cage.

Normally, C varies between 1 and 0. Values of 1 indicate equal competitiveness between sterile and wild males. It is not uncommon to record values of more than 1; therefore, replicates with values greater than 1.1 should be discarded, and values between 1 and 1.1 should be rounded down to 1.0. Values between 0.2 and 0.4 are normal for sterile lab males.

7.6. Field Fried Test

Objective

To provide an estimate of the sterility induced in the field by sterile males.

Discussion

The ultimate measure of the performance of the released sterile males is the reduction in egg hatch in the target population. The Cage Fried test (7.5) provides a good estimate of such sterility induction. However, it is possible to assess it with more detail and directly from the field by recovering live wild females and allowing them to oviposit to collect their eggs. This can be done by placing traps with female attractants that retain the females in the traps but do not kill them. Traps are serviced periodically, and captured females are taken to the laboratory to allow them to oviposit. It is also possible to place oviposition devices in the traps so that females lay the eggs once inside the traps (ref). Percentage of egg hatch can then be compared to that obtained from females captured in areas where no sterile males are released. For the case of *C. capitata*, it has been found that fertility of wild females in those areas can be as high as 90%. In areas under SIT releases, where the wild population is low, the recovery of wild females will be probably very low, and the test will be very labour intensive with probably few results.

Equipment

- Female attractant-baited traps, appropriate for the species being tested. If no female attractant is available, use protein-based or food attractants.
- Oviposition substrate, agar balls wrapped with parafilm or ripe host fruit (free of tephritid eggs).
- Dissecting microscope.
- Dissecting forceps.
- Laboratory oviposition cages.

Procedures

Select one area where sterile releases are being performed and a nearby area where no sterile flies are being released, and the wild population is known to be at high numbers. If no close area is available, set up a control cage as in the standard Fried Test with wild flies. Establish a trap grid as in the Release-Recapture Test or take advantage of the trapping network already established by the action programme. Traps should not contain insecticide and should be adapted so that flies that enter the trap are retained but not killed. Provide a cotton-wick with a water-sugar solution in order to give some nutrients to the flies. Pay special attention to place the traps in areas within the foliage that prevent the traps from direct sun exposure. Check the traps every day and with the aid of an aspirator remove the females. Take them to the laboratory and place them in oviposition cages with adequate conditions to enhance egg laying. Natural fruit can enhance oviposition, but egg collection can be more laborious; artificial domes or agar balls may be inferior oviposition substrates, but eggs are easy to collect. Provide water and food. Collect eggs on a daily basis and place them onto moistened black filter paper in a 9cm Petri dish. Incubate the eggs at 25°C and after 5 days, evaluate the percentage of egg hatch. For meaningful data, collect at least 30-50 females during a period of one week and replicate at least three times during a period of three months (preferably when the wild population is at its peak).

Interpretation

Compute the Fried competitiveness value (C) as described in 7.5. Use the data from an area where no sterile males are released as the control values or replace it by estimates derived from a field cage set up with wild flies. Interpretation of C values should be done as explained for the Cage Fried Test. The main advantage of this test is that it gives the most accurate measure of induced sterility in the field. However, as mentioned before, this test is adequate when the wild population is still at relatively high levels in the area with sterile insect releases. One disadvantage for some species is the lack of a female attractants. This may result in males also entering the trap, which could possibly result in matings. This can be minimized by placing the traps in the field at those times of the day when the flies are not sexually active and removing them before the onset of sexual activity (i.e., late in the afternoon for *C. capitata*, from midday until dusk for *A. fraterculus* or from dawn until the afternoon for some *Bactrocera* species).

Additional information on how sterile males are performing in the field can be obtained by characterizing the sperm present in the spermathecae of wild female with DNA techniques (refsSan Andres and Sabater). This tool has proven to be very sensitive, and it is possible to tell the origin of the sperm, whether the female has mated with a sterile male, a wild male or a male of each type, and other relevant information. However, in order to distinguish wild males from sterile males, this technique requires that the sterile flies released have a genetic background different from that of the target wild population or specific, diagnostic molecular markers exist. In addition, it is also labour consuming and requires sending the material to laboratories that can perform the analysis.

7.7. Survival in Field Cages

Objective

To determine the survival of sterile insects under field cage conditions in the absence of predators.

Discussion

Sterile insects are released in the field where they must survive to achieve matings with wild females. Their survival is therefore one of the most important components for the success of the SIT. The proposed test complements those presented in the Required Periodic Tests and is designed to document sterile male survival under field conditions in order to plan and/or modify production and handling procedures as well as periodicity of the releases.

The test is conducted in the area of release with PVC pipe framed small field cages (1.5 x 1 x 1 m) with a fine mesh. A zipper in the cage floor (**Figure 7.3**) allows introduction of a potted plant of the desired species (e.g., potted coffee plant). Whenever possible, it is recommended that these plants are the same species as in areas where sterile males will be released. Potted plants should be placed outdoors under the same conditions of the release area. This will allow them to collect bird droppings, honeydew, microbes and other sources of nutrition that sterile males will normally find in the field. Prior to the test, potted plants are introduced to the cages. Major effort should be made to assure that there are no predators on the potted plants and that cage is tightly closed to avoid entry of any predators during the test. Small field cages can be placed at locations that are representative of the range of conditions in the release area. Under all these conditions this test should closely approximate the survival of the insects in the field, although excluding effects of predation.

Figure 7.3: Field cage showing zippers on both sides as well as underneath the cage. Bottom opening used to introduce a potted plant. Side openings used for ease of access to collect dead sterile insects.

Source and Handling of Flies

In this case it is recommended that releases in the small field cages use flies that were recovered from the release machine as in the Adult Fliers Post Release Test (see section 5.4.1). If wild flies are to be released in the cages as well, then procedures should resemble those of the Mating Performance Field Cage Test (section 6.2).

Equipment

- PVC pipe framed small field cages (1.5 x 1 x 1 m) with a zipper to allow access into the cage.
- Potted plants, preferably of the same species in areas where the sterile males are released.
- Recording forms.

Procedures

Place the cages at the desired field location (**Figure 7.4**) and recover sterile males from the release machine as proposed in the Adult Fliers Post Release Test. Release the flies inside the cages (100 flies/cage is recommended). Tests may be run with only sterile male flies or as mixed populations of sterile and wild male flies. After 48-72 h (depending on the species) open the lateral zippers and record the number of dead flies on the form. It is advisable to record weather conditions at regular intervals (once an hour) by placing data loggers within the cages.

Figure 7.4: A group of 10 field cages being handled by a single person. Testing is being conducted in a coffee farm.

Interpretation

Perform survival curves with the recorded data in order to determine the mean survival time and the percentage of flies alive at given times. If both sterile and wild males are released, then it is possible to make comparisons of the obtained values. This test can be run as part of the survival control cages in the Release-Recapture Test of Survival and Dispersal (see section 6.3). It is also possible to adjust the frequency at which the cages are checked and to

do it more than once during the test (e.g., every 12 or 24 h) and for a longer period (e.g., one week). Other interesting particular questions that can be addressed with this test are the effect of the diet given to the males before release, the impact on survival of any pre-release treatment (male enhancers, methoprene, etc), and the impact of the release in itself (chilling, handling, etc).

7.8. Pupal Diameter

Objective

To obtain an accurate estimate of the mean size (diameter) of a group of pupae.

Discussion

Diameter is a valuable indicator of overall viability of pupae and correlates with diameter of the resulting adult flies. Larger male tephritids will, in general, be stronger fliers, live longer, have higher mating propensity and induce longer refractory periods in female flies than smaller males. Measured values of mean pupal diameter will vary depending upon the strain and rearing system, so using diameter to compare overall quality of pupae from different facilities must be done with caution. This evaluation should be conducted at the mass-rearing facility.

Equipment

- Balance or scale with accuracy of ± 1 mg or better (**Figure 2.1,** section 2.2.1).
- Soft forceps for handling pupae and removing trash from samples.
- Board with ridges or grooves, or other device for simplifying the process of counting pupae (optional).
- Pupal sizing and separating machine. This consists of two diverging stainless steel cylinders that rotate in opposite directions such that the top of each cylinder moves away from the other cylinder (**Figure 7.5**). The cylinders are on an incline and are aligned so that there is a widening gap between them through which the pupae will eventually fall as they move down the incline. The cylinders can be adjusted to regulate the rate at which the gap widens so that it is possible to collect the pupae into as many as 9 different diameter groups with #1 at the top of the incline being the smallest and #9 at the bottom the largest. A vibrating singulator may be used to deliver pupae individually to the gap at the upper end of the cylinders.
- Small collection containers below each of the chutes of the sizing machine.
- Optical seed counter (optional). An optical counter (**Figure 2.2,** section 2.2.1) may be used for counting pupae in this test, but it must be calibrated to ensure accuracy.
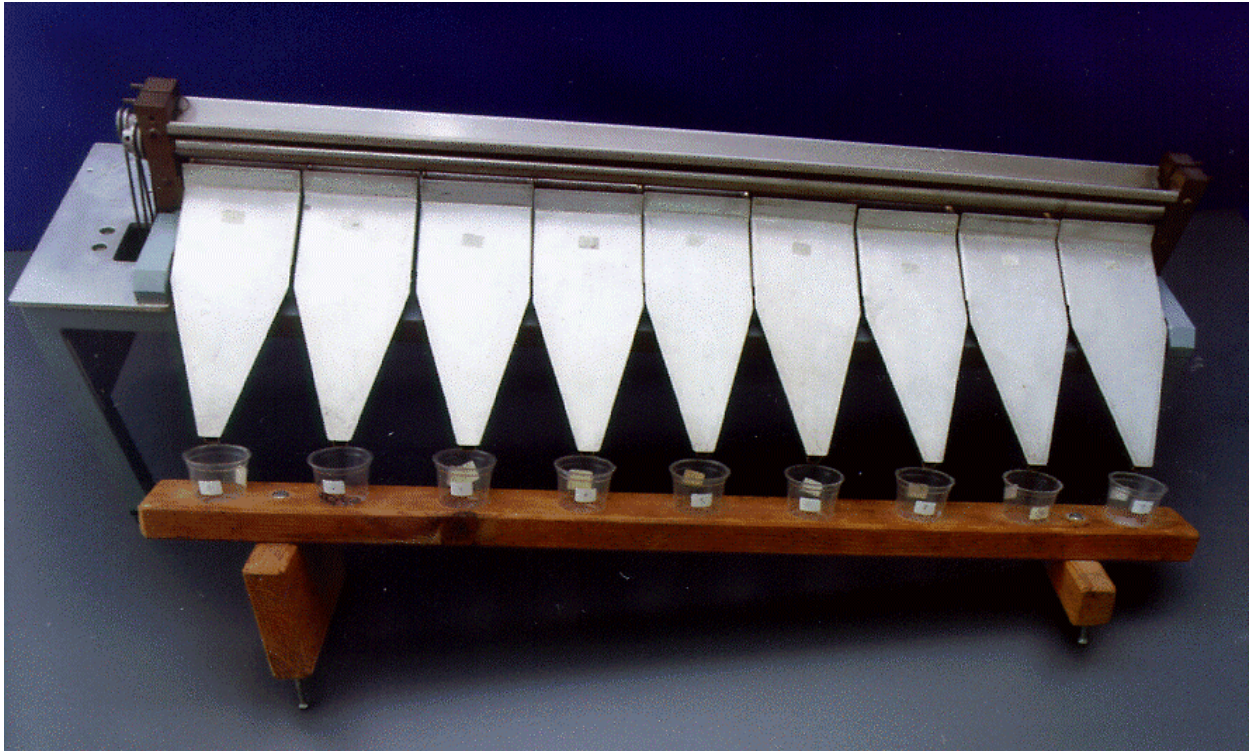
Figure 7.5: Pupal diameter sizing and separating machine.

Procedure

A sample of 500-1,000 pupae is selected volumetrically from the lot to be measured and put through the sizing machine. The singulator should be adjusted, or, if a singulator is not used, extra care must be taken so that pupae fall onto the cylinders individually and do not "bunch up" while going down the incline. As the pupae collected in each group are counted, they should be examined and any that are stuck together or have debris attached to them should be discarded. The numbers of pupae in each group are recorded, and the percentage of pupae in each diameter range is computed. Pupae should be sampled two days before adult emergence.

The gap width between the cylinders from the top to the bottom of the incline will differ for different species:

C. capitata	1.4 mm to 1.9 mm
A. suspensa	1.8 mm to 2.5 mm
A. obliqua	1.3 mm to 2.9 mm
A. ludens	1.3 mm to 2.9 mm

The gap should be calibrated with automotive feeler gauges or by using drill bits of the desired diameter before each use. The cylinders must also be very clean. Contents of the container corresponding to the smallest pupae will include debris but few, if any, pupae and can be discarded.

Interpretation

Downward trends in diameter of pupae produced by a facility can result from poor nutrition, overcrowding in the larval stage or high temperatures in the larval diet. As small diameter is likely to be accompanied by poor performance on other quality indices and in the field it must be avoided by using specific rearing standard operating procedures for each species.

7.9. Survival in the Field

Objective

To estimate survival in the field using data of the number of flies captured in the regular trapping network from the operational programme.

Rationale

During SIT practices in any AW-IPM programme, a trapping network is established and serviced on a regular basis in order to monitor the wild population levels. The amount of information recovered is large, but to date little effort has been done to take advantage of this information as part of the evaluation of the quality of the released males. The test proposed here aims at covering this gap by incorporating slight modifications that allows estimating survival of a given release batch.

Procedures

Coordinate the releasings in a way that two or three consecutive releases are done with flies dyed with different colours (Figure 8.xx). Release the flies immediately after the day the trap was serviced. After a sufficient time in which to guarantee that all flies dyed with a colour different from the one used routinely are dead, repeat this procedure Register the number of flies recovered of each colour from the trapping inspection. Estimate the proportion of flies recovered of each colour (age range) in each trap inspection.

Interpretation

Data will provide information on the survival of flies at the time of routine trap inspection. It can also be used to estimate location of sterile males in the field and whether this is heterogeneous or not and correlate this with that of wild flies. If sufficient replicates are done, any bias originated from some particular areas being harsh or adequate for flies' survival will compensate any under or overestimate of survival. Seasonal impact on survival can also be used to adjust release frequency. In addition, information on the distribution of sterile insects can be used to determine whether sterile males are found in the same traps the wild flies are captured and whether the distribution is heterogeneous as a response to environmental or other factors. Different ways to quantified spatial heterogeneity can be applied such as the coefficient of variation, the spatial autocorrelation, the negative binomial model or the exponent Taylor's power law (see Meats et al. 2006 and references therein).

7.10. Relevant literature

Complement of the relevant literature already suggested at the Required Periodic Tests.

Abraham, S., L. Goane, J.L. Cladera and M.T. Vera. 2011. Effects of male nutrition on sperm storage and remating behavior in wild and laboratory *Anastrepha fraterculus* (Diptera: Tephritidae) females. Journal of Insect Physiology 57: 1501-1509.

Fried, M. 1971. Determination of sterile insect competitiveness. J. Econ. Entomol. 64: 869-872.

Katsoyanos, B. I. and J. Hendrichs. 1995. Food bait enhancement of fruit mimics to attract Mediterranean fruit fly females. J. Appl. Ent. 119: 211-213.

Katsoyanos, B. I., N. Papadopoulos, N. A. Kouloussi, R. Heath and J. Hendrichs. 1999. Method of assessing the fertility of wild Ceratitis capitata (Diptera: Tephritidae) females for use in sterile insect technique programs. J. Econ. Entomol. 92: 590-597.

McInnis, D. O. 1989. Artificial oviposition sphere for Mediterranean fruit flies (Diptera: Tephritidae) in field cages. J. Econ. Entomol. 82: 1382-1385.

Pérez-Staples, D., Aluja, M., 2006. Sperm allocation and cost of mating in a tropical tephritid fruit fly. Journal of Insect Physiology 52: 839-845.

Taylor, P.W., Kaspi, R., Yuval, B., 2000. Copula duration and sperm storage in Mediterranean fruit flies from a wild population. Physiological Entomology 25: 94-99.

Weldon, C. W., J. Preter and P. W. Taylor. 2010. Activity pattern of Queensland fruit fly (Bactrocera tryoni) are affected by both mass-rearing and sterilization. Physiol. Entomol. 35: 148-153

8. Forms for Recording Quality Control Data

8.1. Pupal Weight Assessment Form

Set-up Date: _____

	Shipment Date	Lot #	Sampling Date	Test Date	Tester
☐ Pre Irradiation					
☐ On Arrival					

		Replicate					Sum
		1	2	3	4	5	
	Total Pupae						
	Total Weight						

Calculations:

		Value
$\left\{ \dfrac{\sum\limits_{1}^{5}\text{Replicates}}{\sum\limits_{1}^{5}\text{Total Pupae}} \right\}$	Average Weight *(5 samples)*	

Observations: _____

_____

_____

Authorization: _____

8.2. Emergence and Flight Ability Assessment Form

Set-up Date: _____

	Shipment Date	Lot #	Sampling Date	Test Date	Tester
☐ Pre Irradiation					
☐ Post Irradiation					
☐ On Arrival					

	Elements	Replicate 1	2	3	4	5	Average
T	Number of Pupae						
A	Not Emerged						
B	Part Emerged						
C	Deformed						
D	Not Fliers						

Calculations:

			Value
E	T-(A+B))/T*100	% Emergence	
F	(T-(A+B+C+D))/T*100	% Fliers	
	F/E	Rate of Fliers	

Observations: _____

_____

_____

Authorization: _____

8.3. Survival under Stress Assessment Form

Set-up Date: _____

	Shipment Date	Lot #	Sampling Date	Test Date	Test Time	Tester
☐ Pre Irradiation						
☐ Post Irradiation						
☐ At arrival						
☐ Post Handling/ Chilling						
☐ Post Release						
☐ Post Handling/ Chilling (w/water)						
☐ Post Release (w/water)						

		Replicate					Average
		1	2	3	4	5	
M	Total Males						
DM	Dead at 48/72 hrs						
F	Total Females						
DF	Dead at 48/72 hrs						

Calculations:

			Value
	DM/M*100	% Male Survival	
	DF/F*100	% Female Survival	

Observations: _____

_____

_____

Authorization: _____

8.4. Sex Ratio and Timing of Emergence Assessment Form

A) Sex Ratio

Set-up Date: _____

	Shipment Date	Lot #	Sampling Date	Test Date	Tester
☐ Post Irradiation					
☐ On Arrival					

	Elements	Replicate					Average
		1	**2**	**3**	**4**	**5**	
T	Total Pupae						
M	Male						
F	Female						
A	Not Emerged						
B	Part Emerged						

Calculations:

		Value
M/(M+F)x100	% Male	

Observations: _____

_____

_____

Authorization: _____

B) Time of Emergence

Set-up Date: _____

	Shipment Date	Lot #	Sampling Date	Test Date	Tester
☐ Post Irradiation					
☐ On Arrival					

Hours post hypoxia	Replicate										Sum	
	1		2		3		4		5			
	M	F	M	F	M	F	M	F	M	F	M	F
0												
8												
16												
24												
32												
40												
48												
56												
64												
72												

Calculations:

	24 hours	48 hours	72 hours
Cumulative male emergence at			
Cumulative female emergence at			

Observations: _____

Authorization: _____

8.5. Sterility Assessment Form

Set-up Date: _____

	Shipment Date	Lot #	Sampling Date	Test Date	Tester
☐ Post Irradiation					
☐ On Arrival					

Egg Collection Day		Replicate		
		Control	**Male Sterility Check**	**Female Sterility Check**
1	Number of Eggs			
	Not Hatched			
2	Number of Eggs			
	Not Hatched			
3	Number of Eggs			
	Not Hatched			
4	Number of Eggs			
	Not Hatched			
5	Number of Eggs			
	Not Hatched			
Total	**Number of Eggs (E)**			
	Not Hatched (NH)			

Calculations:

			Control	**Male**	**Female**
	NH/Ex100	% Sterility			

Observations: _____

_____

_____

Authorization: _____

8.6. Mating Performance Assessment Form

Day:_____   Observer/s:_____

Treatment:_____   Cage:_____   Tree type:_____

Male release time: ___   Female release time:_____   End time:___

Fly type	Age at release	Colour	N[1]	Condition[2]
Wild male				
Sterile male				
Wild female				
Sterile female				

[1]State the exact number of flies effectively released in the cage considering replacements. [2]Here state any relevant information regarding the pre-release holding condition and/or treatment such as lot #, shipment date, collection date, host (for wild flies), diet provided, use of enhancers (GRO, methyl eugenol) or hormonal treatment (methoprene), chilling, etc.

Pair #	Start time (hh:mm)	End time (hh:mm)	Mating couple[1]		Location			Observations
			Male	Female	Elevation	Foliage	Quadrant	
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								
18								

[1]**Mating Pair Key**: a pair is always represented by male first and female second. When recording the type of fly, write the colour of it; don't attempt to indicate the origin. [2]**Location Key:** Elevation of mating within the tree at **H**igh, **M**iddle and **L**ow; Position within the foliage (leaf): either **U**nder or **O**ver the leaf or the **C**age; and Quadrant: **NE**, **NW**, **SW**, and **SE** for northeastern, northwestern, southwestern, and southeastern quadrants, respectively).

$\sum SS$	$\sum SW$	$\sum WS$	$\sum WW$

Observations: _____

8.7. Graphic Representation of Indices of Mating Performance

The following graphic form is recommended to represent the values of mating performance indices obtained from mating performance field cage test in a relatively self-explanatory way.

While RSI measures the male sexual competitiveness, the ISI (with FRPI and MRPI) and the RII provides insight on the sexual compatibility (or isolation) between two strains. It is to be noted, that ISI, FRPI and MRPI should always be interpreted and presented together since their meaning is complementary. Data should be plotted in each graph as mean ± standard error).

Male sexual competitiveness

$$RSI = \frac{SW}{SW + WW}$$

RSI

0	0.25	0.5	0.75	+1

All matings by wild males — Equal competitiveness of both males — All matings by sterile males

Strain sexual compatibility

$$ISI = \frac{(SS + WW) - (SW + WS)}{SS + WW + SW + WS}$$

ISI

-1	-0.75	-0.5	-0.25	0	0.25	0.5	0.75	+1

Negative assortative mating — Random mating *(Sexual compatibility)* — Positive assortative mating *(Sexual isolation)*

$$FRPI = \frac{(SS + WS) - (SW + WW)}{SS + SW + WS + WW}$$
$$MRPI = \frac{(SS + SW) - (WS + WW)}{SS + SW + WS + WW}$$

FRPI

MRPI

-1	-0.75	-0.5	-0.25	0	0.25	0.5	0.75	+1

All matings by wild individuals — Equal mating propensity — All matings by sterile individuals

$$RII = \frac{SS \times WW}{SW \times WS}$$

RII

0	+1	+10	+100	+∞

Negative assortative mating — Random mating *(Sexual compatibility)* — Positive assortative mating *(Sexual isolation)*

8.8. Fly Irritability Assessment Form

Day:_____   Observer/s:_____

Cage:_____   Tree type:_____   Fly release time: _____

Fly type	Age at release	Colour[1]	N[2]	Condition[3]
Wild male				
Sterile male				
Wild female				
Sterile female				

[1]Flies should be painted with the fluorescent dye to avoid bias during the test.

[2]State the exact number of flies effectively released in the cage considering replacements.

[3]Here state any relevant information regarding the pre-release holding condition and/or treatment such as lot #, shipment date, collection date, host (for wild flies), diet provided, use of enhancers (GRO, methyl eugenol) or hormonal treatment (methoprene), chilling, etc.

Sampling period	# Wild males	# Sterile males	# Wild females	# Sterile males
Early morning				
Mid-day				
Evening				

Observations: _____

Appendix A: Chronology of Product Quality Control of Tephritid Flies for Use in SIT Programmes

1977 Publication of "An Idea Book for Fruit Fly Workers" (Boller and Chambers 1977), which includes many original papers and bibliographic references on how to measure overall performance, individual traits, and production of laboratory-reared tephritids.

1978 IOBC Palearctic Working Group on Fruit Flies of Economic Importance met at Sassari, Sardinia, Italy where quality control of tephritid fruit flies was proposed as a discipline.

1978 Boller, Calkins, Chambers, Cunningham, Greany, Hendrichs, Huettel, Leppla and Ruhm meet in Guatemala under the auspices of IAEA, USDA, ARS and Moscamed to conduct laboratory and field tests of quality control on laboratory and field collected medflies.

1979 Lek mating system described in Mediterranean fruit fly (Prokopy and Hendrichs 1979). International Organization of Biological Control (IOBC) sponsors a course in Castellon, Spain and a manual (Calkins et al. 1979) to cover methods for assessing pupal size, flight ability, startle activity, olfactometry, mating propensity, dispersal and survival, and ratio tests.

1981 Publication of the RAPID methods and apparatuses for "Measuring, Monitoring, and Improving the Quality of Mass-Reared Medflies" (Boller et al. 1981). This publication suggests that five tests, i.e., pupal size, flight ability, startle activity, response to pheromone, and mating propensity, be carried out at frequent intervals.

1981 An International Technical Group on Quality Control meets in Guatemala to standardize basic methods and tests. Six tests are recommended that should be carried out regularly and nine others as time and resources permit (Klee 1981). Procedures and samples of reporting forms to be used were included.

1982 First mating compatibility test on a field caged host tree, measuring female choice by allowing wild female flies to select among competing wild and sterile mates (Zapien et al. 1983).

1982 IOBC Global Working Group on Quality Control of Mass Reared Insects was formed. The first meeting was held in Gainesville, Florida, USA. Subsequent meetings were held in Wadenswil, Switzerland in 1984, in Guatemala City, Guatemala in 1986, in Vancouver, British, Columbia, Canada,in 1988, Wageningen, The Netherlands in 1991 in Rimini, Italy in 1994, and in Santa Barbara, California, USA in 1996.

1982 International Symposium on Fruit Flies of Economic Importance had a section on Quality Control, Athens, Greece. Also held in 1986 in Crete, 1990 in Antigua, Guatemala, and 1994 in Clearwater, Florida, USA.

1983 Field tests for extending and confirming the results of laboratory data from RAPID tests are published by Chambers et al. (1983). The staff at the Moscamed Programme in Mexico, publishes a manual of >40 laboratory and field tests that have been used extensively to measure the quality of mass produced flies in Latin America (Orozco et al. 1983).

1984 USDA, IAEA and Moscamed Guatemala establish a cooperative pilot test of a systematic process, based on the RAPID tests, for measuring and controlling quality through process control, product evaluation, and data management. A manual for laboratory QC of mass-reared Medflies is developed by C. Calkins and T. Ashley.

1986 USDA-APHIS compiles and distributes "Required quality control tests, quality specifications, and shipping procedures" (Brazzel et al. 1986). This manual was designed to ensure that Mediterranean fruit flies for SIT programmes with USDA involvement meet certain quality standards. The QC tests included were primarily a check on the rearing process and could all be carried out with inexpensive (or easily constructed) equipment in a minimum of laboratory space. This has been the QC guide followed in USDA, Moscamed, and CDFA rearing facilities for the past 10 years.

1989 A case of behavioural resistance (to mating with sterile flies) is documented for the melon fly in Okinawa Islands by Hibino and Iwahashi (1989, 1991). A doubtful case was later reported for the Mediterranean fruit fly from Hawaii (McInnis et al. 1996).

1994 The Insect Pest Control Section of the Joint FAO/IAEA Division for Food and Agriculture launches a Coordinated Research Project to study Mediterranean fruit fly courtship and female choice behaviours, to assess the compatibility of Mediterranean fruit fly populations from different origins world-wide, and to standardize field cage mating compatibility tests.

1996 Indices (*RII*, *RSI*) to measure the mating competitiveness in field cage test are published (McInnis et al. 1996).

1997-98 Nineteen experts from several countries gathered in Vienna for one week to harmonize internationally fruit fly product quality control, and to agree on the manual of "Product Quality Control, Irradiation and Shipping Procedures for Mass-Reared Tephritid Fruit Flies for Sterile Insect Release Programmes". The FAO/IAEA/USDA manual of "Product Quality Control, Irradiation and Shipping Procedures for Mass-Reared Tephritid Fruit Flies for Sterile Insect Release Programmes" (Version 4) is released in September and recognised as an International Guideline for fruit fly SIT projects.

1999 New indices (*ISI*, *MRPI*, *FRPI*) to measure the mating competitiveness in field cage test are published (Cayol et al. 1999). These indices were included in Version 4.0 of the present manual. The Insect Pest Control Section of the Joint FAO/IAEA Division for Food and Agriculture launches a Coordinated Research Project on "Quality Assurance of Mass Produced and Released Fruit Flies" with the objective to further improve and standardise international quality control procedures.

2003 FAO/IAEA/USDA manual of "Product Quality Control and Shipping Procedures for Sterile Mass-Reared Tephritid Fruit Flies" is revised as Version 5 and published. Revised manual includes *required* tests of mating competitiveness and compatibility. In addition, tests were added to provide better information on survival and dispersal of sterile flies in the field.

2005 "Sterile Insect Technique. Principles and Practice in Area-Wide Integrated Pest Management" is published. Textbook contains Chapter on Quality Control. Part III titled "Technology Components of Sterile Insect Technique" contains parts dealing with

behaviour, mass-rearing, sterilizing insects with ionizing radiation, sterile insect quality, insect supply emergence and release, and monitoring sterile/wild insects in an area-wide pest management programme.

2007 FAO/IAEA Coordinated Research Project on Quality Assurance of Mass Produced and Released Fruit Flies is completed. Findings are published as a special issue in the Florida Entomologist. A final report can be viewed at: http://www-naweb.iaea.org/ nafa/ipc/crp/D41016_ev_report_final.pdf . A list of publications resulting from this CRP can be viewed at: http://www-naweb.iaea.org/nafa/ipc/crp/d41016_list_pub _final.pdf

2007 A model for dose optimization for improved sterile insect quality is developed and published.

2007 FAO/IAEA publishes "Guidance for packing, shipping, holding and release of steriles flies in area-wide fruit fly control programmes".

2007 SAGARPA-SENASICA-Direccion General de Sanidad Vegetal Programa Moscafrut issues a new "Manual de Control de Calidad de moscas esteriles y parasitoides: Procedimientos para evaluar el producto (*Anastrepha ludens, Anastrepha obliqua* y *Diachasmimorpha longicaudata*)". This new manual contains both quality control for sterile insects as well as a Mediterranean fruit fly parasitoid.

2007 "Area-Wide Control Insect Pests: From Research to Field Implementation" edited by M.J.B. Vreysen, A.S. Robinson, J. P. Hendrichs is published. This textbook contains papers relevant to SIT programmes and sterile insect quality.

2009 A panel of experts conducts a review of all fruit fly emergence and release facilities supported by the U.S. Department of Agriculture carried out in the United States, Mexico and Guatemala. A final report contains many recommendations for improving efficiency and effectiveness applicable to sterile insect release programmes in general.

2009 FAO/IAEA Coordinated Research Project on Development of Mass Rearing for New World (*Anastrepha*) and Asian (*Bactrocera*) Fruit Flies is completed. Findings are published as a special issue in the International Journal of Tropical Insect Science. A final evaluation report and proceedings will be published soon and can be accessed at: http://www-naweb.iaea.org/nafa/ipc/crp/ipc-mass-rearing.html .

2009 FAO/IAEA Coordinated Research Project on Improving Sterile Male Performance in Fruit Fly SIT programmes is completed. Findings are published as a special issue in the Journal of Applied Entomology. A list of publications produced as a result of this CRP can be viewed at: http://www-naweb.iaea.org/nafa/ipc/crp/d41020-list-pub-final.pdf . A final evaluation report is available at: http://www-naweb.iaea.org/nafa/ipc/crp/d41020-evaluation-report.pdf .

2010 Research is completed and published regarding a new generation X ray irradiators for insect sterilization. These irradiators offer a new alternative to SIT programmes for sterilizing insects particularly where permitting, transport and storage of irradiators with radioisotopes is no longer feasible.

2010 Consultant's meeting is held at the IAEA in Vienna, Austria to begin revising the FAO/IAEA/USDA manual of "Product Quality Control and Shipping Procedures for Sterile Mass-Reared Tephritid Fruit Flies". The newly revised manual will become

Version 6.0. The new manual has been reorganized into two parts: a) mass-rearing facilities and b) fly emergence & release facilities. Required routine and periodic tests are identified for each of the major processes by facility. Absolute fliers post handling/chilling and post release at fly emergence and release facilities were added as required routine tests. Mating performance in field cages and capacity to evade predators were added as a periodic test at mass-rearing facilities. The following required routine tests were renamed: pupal weight (in place of pupal size); survival under stress (in place of longevity under stress). Laboratory mating test has been removed from the manual.

2015 Consultant's meeting is held at the IAEA in Vienna, Austria to begin revising the FAO/IAEA/USDA manual of "Product Quality Control and Shipping Procedures for Sterile Mass-Reared Tephritid Fruit Flies".

Note: the references quoted in this Appendix can be found in the introduction and other sections of this manual.

Appendix B: Known Sources of Key Equipment and Supplies[1]

Item	Source
As described in XX Guidelines for sampling insects for routine QC tests	
Roller gauges [for pupal sizing and separating, ("*puppentransporteinrichtung*")]	**Ing. Alfred PARAL** Postfach 27, Hainfelder Strasse, A-3071 Boeheimkirchen, Austria. Fax: (+43) 274323045
Seed Counter [for counting pupae, ELMOR 600/A05 with screw on conveyor bowl including discharge chute and foot actuator]	**Dr. Rudolf MOLL** Export Department Mangelegg 58, CH-6430 Schwyz, Switzerland. Fax: (+41) 43216508
As described in 0 2.2.2. EMERGENCE AND FLIGHT ABILITY	
Tubing for Flight Ability tubes [Tube-Pak, size No. 52D I.D. 3 ¼", O.D. 3 ½", 8 ft length, Part No. 06048C]	**Consolidated Plastics Company** 9085 Freeway Drive, Macedonia, Ohio 44056, USA.
As described in	
Day-Glo powder [example: Blaze Orange, reference JST43]	**Radiant Color** Europarklaan 80, B-3530 Houthalen, Belgium Tel.: (+32) 11520760, Fax: (+32) 11526679 E-mail: info@radiantcolor.be
Field Cages [20 x 20 HDPE Screen fabric - 2.9 meters diameter x 2.0 meters height with floor and one 2-way zipper (bottom to top)]	**Synthetic Industries** P.O. Box 977, 2100A Atlanta Highway, Gainesville, Georgia, 30503, USA. Fax: (+1) 7705311347
Lightmeter and datalogger [model Testo 545 (lightmeter) and Testostor 171 (datalogger)]	**Testo GmbH & Co.** Postfach 11 40, Testo-Strasse 1, D-79853 Lenzkirch, Germany Tel.: (+49) 7653681-0, Fax: (+49) 7653681-100 E-mail: info@testo.de, Website: www.testo.de
As described in Error! Reference source not found. ERROR! REFERENCE SOURCE NOT FOUND.	

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[1] *Reference to any commercial product or service is made with the understanding that no discrimination is intended and no endorsement by the FAO/IAEA or the United States Department of Agriculture is implied. Reviewers are strongly encouraged to contribute with additional sources of equipment and supplies.*

Item	Source
Attractant [BioLure® Three-Component Fruit Fly Lure (Triple Pack) (trimethylamine, putracine, and ammonium acetate)]	**Suterra** (formerly Consep, Inc.) 213 S.W. Columbia St., Bend OR 97702-1013, USA. Tel.: (+1) 5413172254, Fax: (+1) 5413883705
Attractant [cue-lure]	**Scentry Biologicals Inc.** 610 Central Venue, Billings, MT 59102, USA. Tel.: (+1) 4062485856, Fax: (+1) 4062452790
Attractant [methyl-eugenol (eugenol methyl-ether)]	**American Scientific & Industrial Supplies** PO Box 8247, Radnor PA 19087, USA. Tel.: (+1) 6109647665, Fax: (+1) 6109641860 E-mail: sales@asi-supplies.com **Scentry Biologicals Inc.** *(see above)*
Attractant [torula yeast pellets]	**Scentry Biologicals Inc.** *(see above)*
Attractant [trimedlure]	**Better World Manufacturing Inc.** 5690 E. Dayton Avenue, Fresno CA 93727, USA. Tel.: (+1) 3055958911, Fax: (+1) 3055957806 E-mail: multilure@aol.com **Scentry Biologicals Inc.** *(see above)*
Ultra-Violet Lamp [High Intensity Long-wave. B-100AP]	**Ultra-Violet Products (UVP) Inc.** 2066 W. 11th Street, Upland, CA USA. Fax: (+1) 8004526788 or (+1) 9099463197 E-Mail: uvp@uvp.com European Sales Operations: **Ultra-Violet Products Ltd.** Science Park, Milton Road, Cambridge, CB4 4FH, United Kingdom. Tel.: (+44) 1223420022 E-mail: uvpuk@uvp.com
Ultra-Violet Lamp with magnifier [Philalux II HF, Art. 9865]	**Schwaebische Albumfabrik GmbH & Co.** P.O. Box 60, D-7445 Bempflingen, Germany. Fax: (+49) 712332550

Item	Source
Traps and trapping supplies	**Better World Manufacturing Inc.** *(see above)* **Gempler's Pest Management Supply** P.O. Box 270, 211 Blue Mounds Rd., Mt. Horeb, WI 53572, USA. Tel.: (+1) 8002727672, Fax: (+1) 8005511128 **Scentry Biologicals Inc.** *(see above)* **Sorygar S.L.** (for Tephri® traps) Quinta del Sol n. 37, Las Rozas - Madrid 28230, Spain. Fax: (+34) 916407000 E-mail: sorygar@nexo.es **Suterra** *(see above)*

As described in 0 3.3. IRRADIATION AND Process Control

Item	Source
Dose-specific radiation-sensitive indicators [70, 125, 300 Gy]	**International Speciality Products (ISP) Technologies Inc.** 1361 Alps Rd, Wayne, New Jersey, 07470, USA. Tel.: (+1) 2016284000, Fax: (+1) 2016283016

As described in Error! Reference source not found. ERROR! REFERENCE SOURCE NOT FOUND.

Item	Source
Gafchromic® dosimetry media [type HD-810, package of 5 sheets, 8" x 10 "]	**Elimpex Medizintechnik GesmbH** Spechtgasse 32, A-2340 Moedling, Austria. Tel.: (+43) 2236410450, Fax: (+43) 2236410459 E-mail: falk@elimpex.com (specify "catalog number 37-040") **ISP Technologies Inc.** *(see above)*
Digital Radiachromic® Reader System [model FWT-92D-220]	**Far West Technology Inc.** 330 D South Kellogg, Goleta CA 93117, USA. Tel.: (+1) 8059643615, Fax: (+1) 8059643162

Miscellaneous

Item	Source
Fluorescent microscope [trinocular with phase contrast, model VanGuard 1486FL]	**Service for Science and Industry (SFSI) Inc.** 1101 North Kings Highway, Suite 201, Cherry Hill NJ 08034, USA. Tel.: (+1) 8563210635, Fax: (+1) 8563210636 E-mail: sfsi@sfsi-usa.com
Multi-use temperature and/or humidity monitors	**Temperature Data Systems** Wattstraat 68, P.O. Box 168, 2170 Ad Sassenheim, The Netherlands. Tel.: (+31) 0 252211108, Fax: (+31) 0 252231032 Website: www.temperaturedatasystems.com

Appendix C: Terminology

Accessory glands: glandular structures associated with the spermatheca that produces a material that accompanies the sperm during ejaculation.

Adjacent-1: genetic sexing strains that have a Y-linkage, produce adjacent 1 segregation at meiosis. This produces 2 classes of genetically unbalanced gametes. One class is characterised by a deletion of one part of the Y-linked autosome, while the second class contains the other part of the respective autosome in triplicate. In the latter, a few individuals survive to adulthood. Characteristically, adjacent 1 individuals exhibit lower emergence. Furthermore, there is no evidence that the adjacent-1 individuals reproduce (i.e. in most cases they are too weak to mate and their longevity is reduced dramatically.)

Aedeagus: the male sexual organ used to transfer sperm to the female.

Area: An officially defined country, part of a country, or all or parts of several countries.

Assortative mating: term used to describe a tendency for male/female of a given population to preferably mate with male/female of the same population.

Calling: the act of dispensing pheromone by the male fruit fly to attract the female.

Certificate: An official document which attests to the phytosanitary status of any consignment affected by phytosanitary regulations.

Classical biological control: The intentional introduction and permanent establishment of an exotic biological agent for long-term pest control.

Compatibility (mating): term used when females of a given strain are able and willing to accept, for mating, the males of another strain; this also includes synchrony and other factors that cause reproductive disconformancy.

Competition: interaction between organisms that share a limited environmental resource.

Competitiveness: ability of an organism to compete with conspecific organisms for a limited environmental resource.

Consignment: a quantity of plants, plant products and/or other biological articles being moved from one country to another and covered, when required, by a single phytosanitary certificate (a consignment may be composed of one or more commodities or lots).

Consignment in transit: a consignment which is not imported into a country but passes through it to another country, subject to official procedures which ensure that it remains enclosed, and is not split up, not combined with other consignments nor has its packaging changed.

Contaminating pest: a pest that is carried by a commodity and, in the case of plants and plant products, does not infest those plants or plant products.

Control (of a pest): Suppression, containment or eradication of a pest population.

Control chart: to plot a parameter with predetermined limits on a time scale and to present this information in an easy to interpret graphical form such as on mean- or range-charts that have control limit lines.

Copulation: a sexual union.

Courtship: the courting behaviour of male animals with the expectation of mating.

Data logger: a device used to record temperatures (or any other environmental variable) during a variable length of time.

Deformed (wings): wings of flies that are not fully expanded or are bent or crumpled.

Detection survey: Survey conducted in an area to determine if pests are present.

Diel periodicity: time of day at which an organism tends to exhibit a behaviour or trait.

Emergence (adult emergence): the escape of the adult insect from the cuticle of the pupa.

End user: the agency or personnel that actually uses or releases the flies received from the producer.

Entry (of a pest): Movement of a pest into an area where it is not yet present, or present but not widely distributed and being officially controlled.

Entry (of a consignment): Movement through a point of entry into an area.

Eradication: Application of phytosanitary measures to eliminate a pest from an area.

Establishment: Perpetuation, for the foreseeable future, of a pest within an area after entry.

Exotic: Not native to a particular country, ecosystem or eco-area (applied to organisms intentionally or accidentally introduced as a result of human activity). As this Code is directed at the introduction of biological control agents from one country to another, the term "exotic" is used for organisms not native to a country.

Flight ability: adult capability to achieve a defined flight performance.

Hazard: Elements or events which represent potential harm; an adverse event or adverse outcome (based on OIE definition).

Hazard (phytosanitary)**:** Injury or deleterious effects caused by pests to plants, plant products or the health of plants in an ecosystem.

Incursion: Presence of a pest population within an area where it is capable of causing economic damage but not capable of establishment. (based on Art VII.3 of IPPC text).

Irradiation certificate: document presented by the shipper to the importer to certify that the insects contained in the package were irradiated at a specified dose.

Lek: a communal display site where males aggregate for the sole purpose of attracting and courting females and to which females come for mating.

Lux: a unit of illumination equal to one lumen per square meter. The lumen is ca 1/683 Watt.

Mating pair: male and female flies in copula. This does not obviously include sperm transfer.

Mating system: the process of assuring that males and females of a given species interact sexually for reproduction. In the case of pest tephritid fruit flies, it is a female choice system.

Normal non-flier: a fly that appears to be normal but does not fly.

Occurrence: The presence in an area of a pest officially recognized to be indigenous or introduced and/or not officially reported to have been eradicated.

Outbreak: An isolated, recently detected pest population.

Over flooding ratio: the ratio of sterile flies to wild flies in the population in an SIT programme.

Parasite: An organism which lives on or in a larger organism, feeding upon it.

Parasitoid: An insect parasitic only in its immature stages, killing its host in the process of its development, and free living as an adult.

Pathogen: Micro-organism causing disease.

Pest: Any species, strain or biotype of plant, animal or pathogenic agent injurious to plant or plant products.

Pest risk analysis: The process of evaluating biological or other scientific and economic evidence to determine whether a pest should be regulated and the strength of any phytosanitary measures to be taken against it.

Pest risk assessment: Evaluation of the probability of the introduction and spread of a pest and of the associated potential economic consequences.

Pest risk management: Evaluation and selection of management options to reduce the risk of introduction and spread of a pest.

Pest status (in an area): Presence or absence, at the present time, of a pest in an area, including where appropriate its distribution, as officially determined using expert judgement on the basis of current and historical pest records and other information.

Phytosanitary action: An official operation, such as inspection, testing, surveillance or treatment, undertaken to implement phytosanitary regulations or procedures.

Phytosanitary certificate: Certificate patterned after the model certificates of the IPPC.

Phytosanitary measure: Any legislation, regulation, or official procedure having the purpose to prevent the introduction and/or spread of pests.

Phytosanitary procedure: Any officially prescribed method for implementing phytosanitary regulations including the performance of inspections, tests, surveillance or treatments in connection with regulated pests.

Point of entry: Airport, seaport or land border point officially designated for the importation of consignments, and/or entrance of passengers.

Process control: regulation of the performance of production processes through feedback so that deviations from product tolerances and specifications do not occur. Parameters within the realm of process quality control for fruit fly production include, among others, percent egg hatch, eggs per unit of diet, quantity of diet inoculated, percent pupation, age of pupae irradiated.

Production control: development, installation, and maintenance of methods used to produce a product at the greatest rate, most efficiently.

Product control: the composite product characteristics of production and testing to which the product in use will meet the expectations of the customer. Parameters within the realm of product quality control for fruit fly production include, among others, pupal weight, sex ratio, longevity, flight ability, pheromone production and response, mating propensity, and mating compatibility.

Pheromone: a chemical produced by one organism that influences the behaviour of another organism of the same species.

Partially emerged: a fly that has not completely emerged from the pupal case, ranging from only the head free to the case adhering to the abdomen.

Packing: the act of placing the pupae into a package and the package placed in a shipping container prior to shipping.

Propensity: an inclination or tendency; the tendency for an individual insect to carry out an act, or for an individual event to occur.

Quality: the degree to which a product meets the requirements of the objective or of the expected function.

Quality control: a systematic process where by management(s) critically evaluates the elements of production, establishes standards and tolerances, obtains, analyses, and interprets data on production and product performance, and provides feedback so as to predict and regulate product quality and quantity.

Range: the area between limits of variation especially as representing a scope of effective operation.

Recombination: the genetic exchange between two homologous chromosomes leading to the occurrence of recombinants. In case of genetic sexing strains, it refers primarily to recombination in males resulting, in the next generation, in a reversal of the sexing system, i.e. recombinants are either wild-type females or mutant males.

Release (into the environment)**:** Intentional liberation of an organism into the environment (see also introduction and establishment).

Release (of a consignment): Authorization for entry after clearance.

Remating: the act of a male or female mating again at some time after previous mating.

Refractory period: the period between matings, usually induced by a substance from the male that inhibits the female from mating.

SOPs: refers to various Standard Operating Procedures (however, each facility has also its SOPs for process qc, etc.) developed by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture. The SOPs may be entitled as such or appear within other publications such as Quality Control Manuals, but must be recognized by this international organization.

Specifications: possessing or concerned with properties that characterize a factor used to describe a quality aspect.

Sperm transfer: the successful transfer of sperm from a male to a female spermathecae during copula. This may be accompanied by accessory gland fluid.

Standard: a quality or measure serving as a basis or principle by which others conform or should conform or by the accuracy or quality of others is judged.

Sterility (radiation induced): A condition in which sperm or eggs from irradiated reproducing individuals do not result in fertile offspring following fertilization.

Strain: a breed or stock of fruit flies that have been held in isolated colonies for a period of time.

Suppression: The application of phytosanitary measures in an infested area to reduce pest populations.

Surveillance: An official process which collects and records data on pest occurrence of absence by survey, monitoring or other procedures.

Survey: An official procedure conducted over a defined period of time to determine the characteristics of a pest population or to determine which species occur in an area.

Target population: the wild population that the sterile flies are being released against.

Treatment: Officially authorized procedure for the killing, removal or rendering infertile of pests.

Wild fly: a fly that has never been domesticated or held in a rearing colony.

Additional terms can be found at the Glossary of SIT terms (http://nucleus.iaea.org/ididas/ SITGlossary.aspx).

Appendix D: Transboundary Shipments of Sterile Insects

Prepared by an FAO/IAEA Consultants Group
30 July to 3 August 2001, Vienna, Austria

PREAMBLE

A Consultants Group Meeting was held to discuss the potential risk[1] from transboundary[2] shipment of sterile insects for pest control programmes. This meeting took place in Vienna at the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, from 30 July through 3 August 2001. The group of consultants (see Annex 1) was called together in response to requests for guidance from national plant protection organizations (NPPOs) in light of the growing demand for alternatives to pesticide use as an exclusive control measure and the increasing interest from the private sector to invest in the Sterile Insect Technique (SIT).

The aim of the meeting was to characterize the potential risk posed by transboundary shipment of sterile insects shipped for SIT programmes and to reach conclusions regarding the level of risk. In the process of this analysis, the group identified some routinely applied procedures, including best practices for shipment that reduce the risk to a negligible level. However, there currently are no internationally recognized guidelines for regulating shipment of sterile insects.

Harmonized guidance regarding regulation of the shipment of sterile insects will facilitate trade while addressing concerns about shipment of what could be quarantine pests. This document was developed as a discussion paper for consideration by the Interim Commission on Phytosanitary Measures (ICPM), the governing body for the International Plant Protection Convention (IPPC).

One possible result of this discussion paper will be the development of an international standard providing guidance on measures pertaining to the transboundary shipment of sterile insects. Alternatively, this topic could be added to the International Standard on Phytosanitary Measures (ISPM) regarding biological control agents (IPPC, 1996) at the time of its revision. However, certain provisions in the ISPM on biological control agents are inappropriate when considering sterile insects (e.g. holding in quarantine for the next generation). In addition, the IPPC Glossary of Terms (IPPC, 2001) definition of biological control excludes the SIT.

In the interest of harmonization, similar discussions may be needed at the Office International des Epizooties (OIE) and the World Health Organization (WHO) regarding the use of sterile insects for control of human or animal diseases.

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[1] "Risk" in this context includes both the likelihood and the consequences of an adverse event occurring

[2] "Transboundary" in this context refers to entry (Customs and Agriculture clearance) of a shipment into the importing country as well as transit shipment through a third country. Transit may or may not involve transloading.

- The increased use of the Sterile Insect Technique (SIT) to suppress or eradicate insect pest populations is resulting in increased shipment of the sterile target insect pests from one country to another, often passing in transit through other countries. These transboundary shipments are not subjected to international standards for biological safety.

- As the SIT becomes more commercial, the need for guarantees that the sterile insects can be safely and legally shipped are essential to encourage financial investments in commercial sterile insect mass-rearing facilities. Also, international regulations are required to reduce the need for independent development of national regulations that may hinder the insect control programmes.

- The objective of the Consultants Meeting was to prepare a discussion paper for consideration of the Interim Commission on Phytosanitary Measures (ICPM), the governing body for the International Plant Protection Convention (IPPC), as a first step towards developing an international standard or other guidance on the transboundary shipment of sterile insects. Additional discussions may be needed to address shipments of sterile insects for control of pests of veterinary and medical importance.

- The scope of the discussions was limited to radiation-sterilized insects for use in Sterile Insect Technique (SIT) control programmes against plant insect pests. Insect strains produced artificially by genetic engineering or other modern biotechnology methods were excluded.

- Four potential hazards were identified with regard to transboundary shipments of sterile insects:

  1. Outbreak of the target pest in a new area, where it does not already occur.
  2. Increase of fitness of the local pest population through the introduction of genetic material from the escaped insects into an area where the pest already exists.
  3. Unnecessary regulatory actions being initiated following false identification of captured sterile insects and conclusion that it is a quarantine threat.
  4. Introduction of exotic contaminant organisms in a shipment, other than the target species for the SIT programmes.

- Transboundary shipment of sterile insects has taken place on a continuous basis for nearly 50 years. The total number of sterile insects shipped was estimated at 962 billion in more than 12,000 shipments to 22 recipient countries from 50 sterile insect factories in 25 countries. During this long period and many precedents, no problems associated with the hazards listed above or any other have been identified, and thus the shipment of sterile insects have never been subjected to any regulatory action.

- The potential risks of the identified hazards were evaluated using a scenario analysis technique.

- The events considered for hazard 1, were: sterilization failure, shipment packages opened accidentally, escape, survival and reproduction of the sterile insects. For hazard 2, in addition to the above sequence of events, the escaped insects would have to reproduce

with a local population and undesirable traits established in the population. For hazard 3, the critical points would be shipment packages opened accidentally, escape, survival and captured insects not recognized to be sterile. Hazard 4 is not unique to sterile insects and was thus not assigned a risk, as it is possible in shipments of goods of any type.

- For each hazard the calculated estimated risk was:
    1. 0.5×10^{-18}
    2. 0.5×10^{-23}
    3. 1×10^{-11}
    4. Many-fold less likely than the risk of moving biological control agents

- It was concluded by the consultants that the present systems of transboundary shipment of sterile insects for SIT programmes is very safe. However, international regulations should be developed for approval by the Interim Commission on Phytosanitary Measures (ICPM) to facilitate commercial development of the SIT.

I. INTRODUCTION

There is a growing demand for cost effective control of insect pests of plants, as well as insects of veterinary and medical importance. At the same time insecticides are under greater scrutiny for potential toxicological and environmental impacts. An alternative insect pest control method is the Sterile Insect Technique (SIT). This involves mass-production of the target insect species, sterilization using ionising radiation and repeated release into the target population. The release of sterile insects that target a population of the same species is a form of "birth control". The sterile insects mate with the wild population but fertilization results in no viable offspring. Repeated releases of sterile insects lead to a reduction in the pest population.

The SIT differs from classical biological control, which involves the introduction of exotic biological control agents, in the following key areas:
1. Sterile insects are not self-replicating and cannot become established in the environment.
2. Autocidal control is by definition intraspecific.
3. SIT used against an established pest never introduces an exotic species into the ecosystem where the SIT programme is being implemented.

The SIT has been used for nearly 50 years for eradication, suppression and control programmes of both plant and animal pests (e.g. Mediterranean fruit fly (medfly, *Ceratitis capitata*) and New World screwworm (NWS, *Cochliomyia hominivorax*). Because of the limited number of facilities for rearing and sterilization, sterile insects are often shipped for release in other locations. Transboundary shipments have gone from production facilities to release sites in countries throughout the world. Demand for SIT is rising and new commercial facilities may be constructed soon to meet this demand.

I-A   Background on transboundary shipments

Transboundary shipments of sterile insects have been made on a continuous basis for the past 46 years. The first shipment of sterile NWS was from its production site at the USDA/APHIS mass-rearing facility in Florida, USA, to the Caribbean island of Curaçao in 1954. This effort resulted in the eradication of the NWS from the island that same year. This was the first eradication of an insect pest population using the SIT.

Most of the transboundary shipments of sterile insects have originated from production facilities in North and Central America for shipment to at least 22 countries in 4 continents including the Americas, Europe, Africa and Asia (see Annex 3). One example is the ongoing shipment of sterile Mediterranean fruit fly pupae from the production factory in Tapachula, Chiapas, Mexico, to the packing and emerging facility in the southwest of Guatemala. Since 1979, biweekly ground and air shipments have been carried out amounting to 280 billion sterile flies (ca. 4,830 tons) in 21 years. Another important case is the ground and air shipment, since 1992, of 104 billion sterile NWS (ca. 1,733 tons) from the screwworm factory in Tuxtla Gutierrez, Chiapas, Mexico, to all of Central America, Panama and the Caribbean.

In Europe, most transboundary shipments of sterile insects have been carried out in support of SIT pilot projects. The first case involved sterile Mediterranean fruit flies shipped from the FAO/IAEA Agriculture and Biotechnology Laboratory in Seibersdorf, Austria, to the island of Procida, Italy, in 1970. There are some other examples of transboundary shipments of sterile insects produced in Europe such as the case of the 206 million sterile Mediterranean fruit flies shipped from the mass-rearing facility in Madeira, Portugal to Israel during 1997/98.

Other cases involving Europe include transit shipments of sterile pupae from Guatemala, Central America, through Amsterdam, Frankfurt or Madrid, to Israel and South Africa and from Mexico, through Frankfurt, to Libya, (see Appendix E).

In the past 46 years, at least 962 billion sterile insects (equivalent to about 18,000 tonnes) have been shipped domestically and internationally. None of these shipments has ever been prohibited from transit or entry for phytosanitary reasons by the 22 recipient countries or numerous transiting countries. The sterile insects are shipped by air cargo (commercial airlines or charter planes) or by ground in refrigerated trucks. They are packed in labelled, sealed containers to prevent contamination or escape. These safeguards are in place to protect the integrity of the sterile insects and not that of the public, property or the environment in the event of a massive escape. The same measures serve as safeguards against the hazards identified in this document, however, thereby greatly reducing any risk.

I-B   Existing Guidelines

Internationally recognized guidelines on many steps in the mass-rearing and sterilization of insects and quality control (materials used in production, the product and process) already exist (see References Section IX) but there are no internationally recognized guidelines for regulating shipment of sterile insects. Some countries do not regulate shipment of sterile insects, others only require labelling and documentation, and still others are regulating sterile insects under their biological control measures. In order to encourage a harmonized approach to national treatment of this method of plant pest control, some guidance on the risks involved will be very useful.

II. SCOPE

This discussion paper characterizes the risks involved with the transboundary shipment and importation (either in-transit through third countries or directly to the importing country) of sterile insects for use as autocidal control agents in control programmes of plant insect pests. Mass-production site hazards and risks related to the release of sterile insects did not fall within the terms of reference of this Consultants Group.

Shipment of sterile, mass-reared insects was considered including those developed through traditional selection and mutation breeding, for example sexing strains. Sterile insects resulting from strains which may be created artificially by genetic engineering or other modern biotechnology methods were excluded.

This discussion paper is also limited to the shipment of sterile insects resulting from radiation-induced sterility and does not deal with sterile insects resulting from the application of other sterilization techniques (e.g. chemosterilants or transgenically-induced sterilization).

III. HAZARD IDENTIFICATION

A key objective of the Consultants Group was to identify and characterize potential phytosanitary hazards associated with the transboundary shipment of sterile plant insect pests. The Consultants identified hazards and distinguished independent events leading to the occurrence of each hazard. This provided a format for estimating the likelihood and characterizing the consequences of each hazard in a scenario analysis[3]. **Figure 1** shows the scenarios for each of the hazards.

Four potential hazards were identified as follows:

HAZARD	PRIMARY EVENT THAT COULD RESULT IN THIS HAZARD
1. Outbreak of target insect pest in a new area	Faulty sterilization
2. Increase of fitness of local pest population	Faulty sterilization
3. Unnecessary regulatory action initiated	Faulty ID of sterile insect
4. Introduction of exotic (new) contaminant organisms	Presence of hitch-hikers in shipments

The first two scenarios require failure of the sterilization treatment as the first event. This could mean absolute failure (i.e. the shipment was not treated) or that the treatment was less than necessary to meet the required specifications for sterility.

The second event that must occur in the first two scenarios is a breach of the package to allow for spillage or escape. It is assumed that in most situations this will be under adverse conditions (e.g. airport cargo handling environment). As a result, the pest must not only be liberated (event c), but it must also survive to escape into a favourable environment (event d).

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[3] Reference for scenario analysis technique (L. Miller et. al., 1993).

Finally, it must mate and reproduce for either hazard 1 or 2 to occur. However, in the case of hazard 2, the scenario recognizes that the introduction of new genetic material in itself does not present a risk unless an undesirable genetic trait is expressed and also has a selective advantage to become established in the population (event e).

The situation in hazard 3 is not related to biological consequences but rather based on regulatory actions (e.g. delimiting survey) that may be unnecessarily taken by the country where the pest is detected but not recognized as sterile. Adverse phytosanitary measures may be put in place by trading partners based on reporting the detection without distinguishing the pest as sterile.

Hazard 4, the introduction of exotic contaminating organisms, was not characterized in the same way as the other three hazards because it is a complex set of sub-scenarios depending on the nature of the contaminant organisms (e.g. parasitoids, virus, etc). This hazard is also different because it is not unique to sterile insects. Similar hazards exist with shipment of biological control agents and to some extent with any shipment. In fact, the sterile insect mass-rearing process virtually eliminates any parasitoids.

In each of the three scenarios (hazards 1, 2 and 3) for which independent events were identified, the likelihood of each event occurring is represented by rough estimates of the probability (a point estimate). The product of the estimates for independent events in each scenario gives an overall estimate for the probability of the hazard occurring. It is noted that the mathematical relationship of these events means that where any event in a scenario is zero, the probability for the entire scenario is also zero.

The estimates are based on data, past programme records, and experience and expert opinion, primarily as regards fruit fly and some Lepidoptera species. They involve extremely rare events for which the primary source of evidence is the substantial history of experience with SIT shipments since 1954 and detailed knowledge of the technical/scientific aspects of the technology.

This approach was used to allow the comparison of risk levels between events and hazards associated with the transboundary shipment of sterile insects. It was not intended to be quantitatively precise, but more importantly to clarify the relative differences in magnitude. It is also useful to facilitate the comparison of phytosanitary risks associated with the transboundary shipment of sterile insects with those associated with other transboundary shipments (e.g. biological control agents).

The scenario analysis process is limited to characterizing direct phytosanitary hazards associated with the range of insect plant pests historically and currently controlled by SIT for phytosanitary applications. It should be noted that the scenarios are useful for pest risk management to the extent that they help to distinguish control points where risk-reducing measures may be applied.

The process does not consider indirect hazards or evaluate the risks against the benefits (e.g., increased pesticide use without SIT). In particular, it should be recognized that although the level of risk for any particular hazard may be the same for an importing and transit country, the transit country does not benefit to the same degree as the importing country from

accepting this risk. In any case measures decided by either importing or transit countries should be technically justified (based on risk analysis or an international standard).
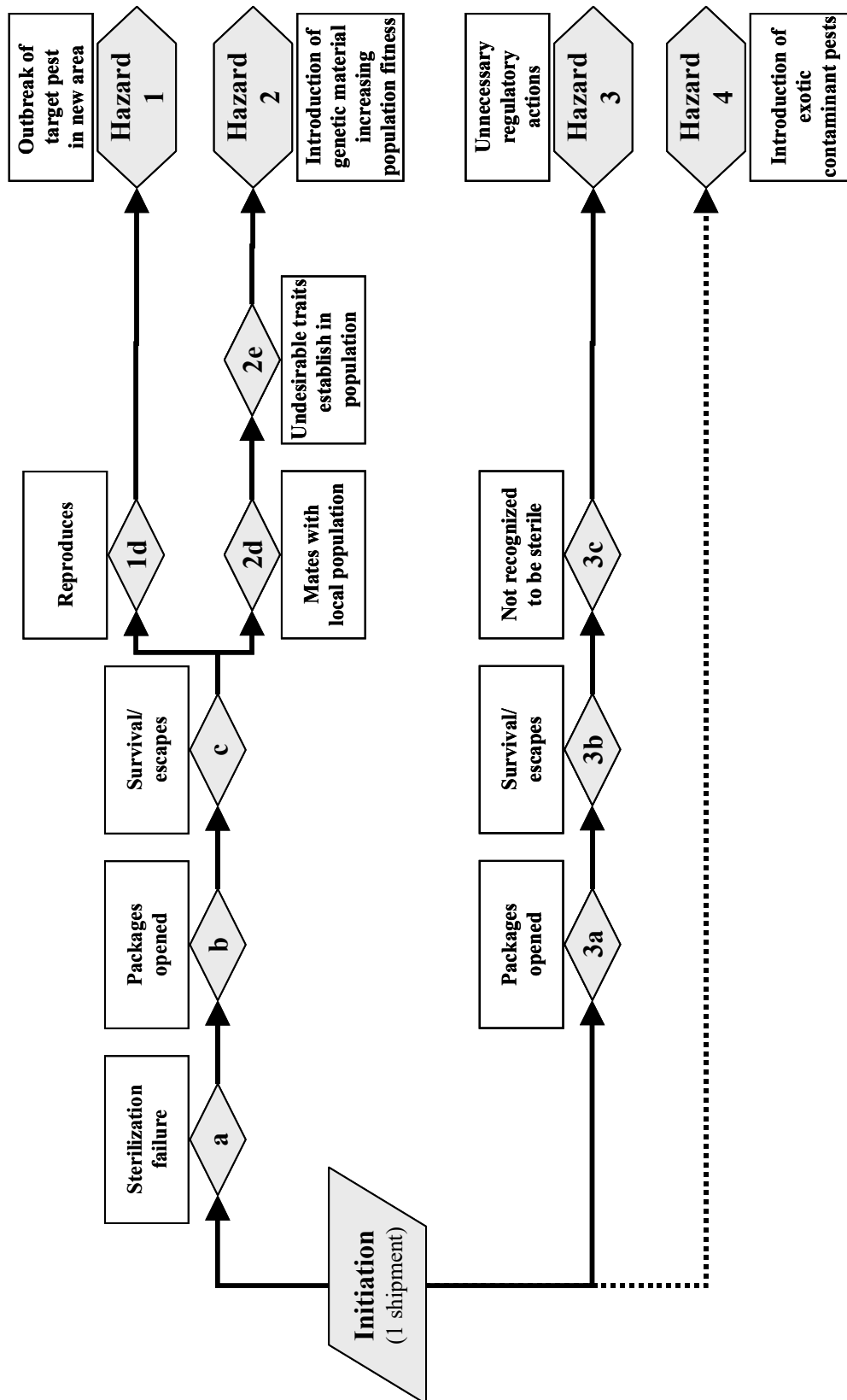

Figure 1. Hazard Scenarios for Transboundary Shipment of Sterile Insects.

IV. LIKELIHOOD OF THE EVENT

IV-A Hazard 1: Outbreak of the target insect pest in a new area

Event *a*: Sterilization failure

An estimated 12,000 ground and air shipments of sterile insects have occurred since 1954 and two instances of partial failure to sterilize (1 confirmed and 1 unconfirmed) have been reported. The confirmed incident occurred in 1982 in a shipment of medflies from Costa Rica to Guatemala (S. Sanchez, personal communication, 1982) and the unconfirmed incident with a shipment of medflies from Peru to California, USA, in 1980 (Rohwer, 1987). Since then, international quality control standards were put in place and there have been no sterilization failures despite the significant increase in the use of SIT.

Current safeguards to prevent sterilization failure:

- Modern production facilities employ failsafe irradiation systems (i.e. physical and/or procedural) to prevent this.
- Each treated container has a dosimetry device that assures the container was irradiated.
- Minimum dosage received by all the insects far exceeds the dosage required to sterilize the females.
- Irradiators are equipped with automatic exposure settings that are tamper-proof.
- Procedures are observed for routine calibration of the equipment.
- Packages are clearly labelled as containing irradiated insects.
- A sample of insects from each shipment is bio assayed for sterility at factory and release site for quality control.

*The likelihood was estimated by the consultants group to be an **extremely rare event** with an estimated probability of **0.5×10^{-6}***

Event *b*: Packages open

In addition to the above event, it would be unlikely for the packages carrying the fertile insects to open because:

- From tens of thousands of containers shipped since 1954 there has been no documented case of breakage of shipping package.
- Using one of the longest routes (i.e. Guatemala City-Miami-Frankfurt-Tel Aviv) from 1998 to 2001, 1 out of over 400 shipments was never recovered. In this event, due to the length of time involved, highly perishable material (i.e. sterile insects) would not survive.
- Current safeguards to prevent mishandling leading to breakage of package include:
    - All consignments are double packaged, some triple packed, and then sealed.
    - Consignments are closely tracked with commercial motivation for rapid transit of highly perishable material.
    - Rapid feedback from receiver when the package is delayed.
    - Size and weight of package designed to minimize breakage.
    - All packages are appropriately labelled (e.g. fragile, biological material) and numbered.

- Content of package does not attract theft.

*The likelihood was estimated by the consultant group to be an **extremely rare event** with an estimated probability of 1 **x 10⁻⁵***

Event *c*: Survives/escapes

In addition to the above events, the fertile insects would be unlikely to survive and disperse to a favourable habitat because:

- Immediate in-transit area is inhospitable (i.e. lack of water, food, wrong temperature, no host, concrete/asphalt substrate). Presence of insecticide/toxicants at airports.
- Airport security prevents unauthorized removal of packages from the airport.
- Limited survival from pupal to adult stage, and even lower chance to survive to sexual maturity and disperse because of high predation, desiccation, starvation, drowning, temperature stress, etc.

*The likelihood was estimated by the consultant group to be a **fairly unlikely event** with an estimated probability of 1 **x 10⁻³***

Event *d*: Reproduces

In addition to the above events, reproduction by the escaped insects would be unlikely because:

- Event may occur during seasonally inhospitable period.
- Climatic factors not suitable for establishment.
- Factory strain has lower fitness for survival in nature.
- Too few survivors to disperse and find suitable environment, mating partners and hosts.

*The likelihood was estimated by the consultant group to be a **rare event** with an estimated probability of 1 **x 10⁻⁴***

For the scenario for hazard 1 the likelihood of all four events occurring was estimated as a negligible risk with a probability of 0.5 x 10⁻¹⁸

Summary of hazard 1: Outbreak of the target insect pest in a new area
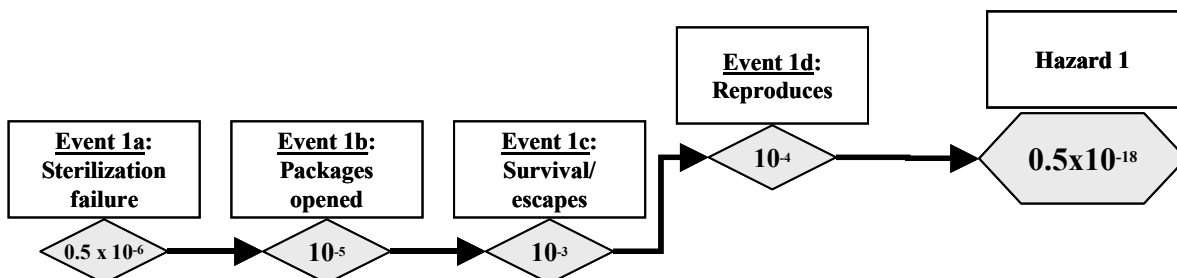
IV-B Hazard 2: Increase of fitness of the local pest population through introduction of genetic material from the escaped insects

For this scenario to take place, events 2a, 2b and 2c must occur. These have the same values as 1a, 1b and 1c. In addition, events d and e must occur:

Event d: Escaped insects reach sexual maturity and mate with local population

In addition to the above events, the escaped insects would be unlikely to reach maturity and mate. This event is very similar to *1d* but assumes that an established pest population exists in the area and that wild mates are receptive to mating.

*The likelihood was estimated by the consultants group to be a **fairly unlikely event** with an estimated probability of **1×10^{-3}**.*

**Event e: Undesirable traits established in the population**

In addition to the above events, the escaped insects would have to possess traits that convey a selective advantage leading to increased fitness. Furthermore, these traits would have to become established in the population. However, this is extremely unlikely because:

- Most introductions of genetic material have neutral or even a detrimental effect on the population. Furthermore, because of the small numbers of escaped insects, it is unlikely that these traits would become established in the wild population.

- Under mass-rearing conditions over many generations, all laboratory strains are known to loose their fitness to survive under natural conditions, therefore they are highly unlikely to carry genetic traits that would increase the fitness of the wild population.

- In addition, the only known traits that have been introduced into mass-reared strains through traditional selection and mutation breeding (i.e. markers and sexing features) are detrimental (e.g. temperature sensitive lethal).

*The likelihood was estimated by the consultants group to be an **extremely rare event** with an estimated probability of **1×10^{-6}**.*

For scenario 2 the likelihood of all five events occurring was estimated as a negligible risk of 0.5×10^{-23}

Summary of hazard 2: **Increase of fitness of the local pest population through introduction of genetic material from the escaped insects.**
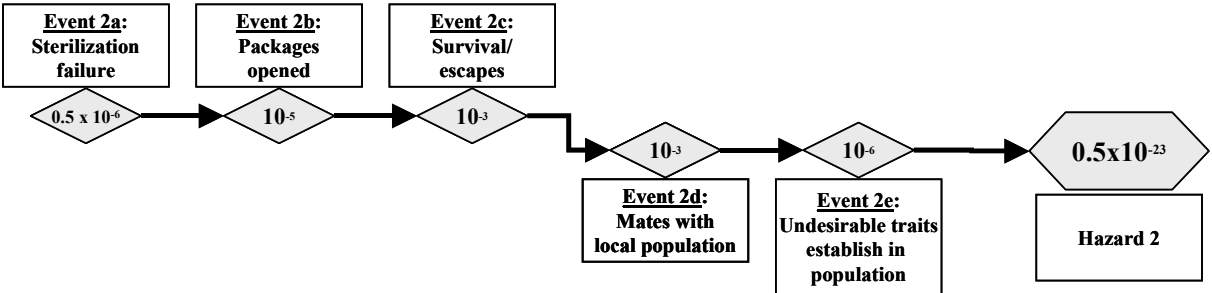
IV-C Hazard 3: Unnecessary regulatory actions initiated due to failure to recognize the detected insect as sterile

Event 3a (i.e. packages opened) is identical to event 1b. Event 3b (i.e. survives and escapes) is the same as event 1c.

Event c: Not recognized to be sterile

In addition to the above events, the escaped insects would have to be detected and not recognized as sterile.
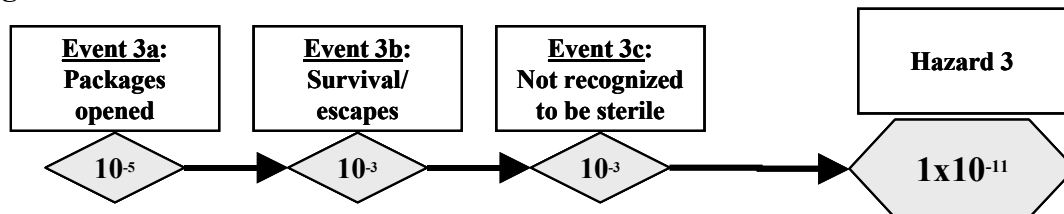
For this to occur the insect must be of regulatory significance:

- The plant protection authorities would have to be conducting detection surveys.
- The plant protection authorities would have to fail to recognize that this could be a sterile insect, which is an unlikely event. Those countries that are most likely to take a regulatory action have standard operation procedures that recognize the possibility of capturing sterile insects.
- The sterile insect marking process and cytological identification for sterility would have to fail.

*The likelihood was estimated by the consultant group to be a **fairly unlikely event** with an estimated probability of 1×10^{-3}.*

For scenario 3 the likelihood of all three events occurring was estimated as a negligible risk of 1×10^{-11}.

Summary of hazard 3: Unnecessary regulatory actions initiated due to failure to recognize the detected insect as sterile

Event 3a: Packages opened	Event 3b: Survival/ escapes	Event 3c: Not recognized to be sterile	Hazard 3
10^{-5}	10^{-3}	10^{-3}	1×10^{-11}

IV-D Hazard 4: Introduction of exotic (new) contaminant organisms

The introduction of exotic contaminant organisms was characterized in a different way because of the complexity of the sub-scenarios involved depending on the nature of the contaminant organisms (e.g. parasitoids versus micro-organisms). This hazard is also different because it is not unique to sterile insects. Similar hazards exist with shipment of biological control agents and to some extent with any shipment. Therefore it was compared to the risks from the shipment of biological control agents, which is widely practiced.

The risk of sterile insect shipments introducing exotic organisms were estimated to be considerably smaller based on the following considerations:

- There is no documented evidence that such an event has occurred during the past 46 years of sterile insect shipping.
- The items being shipped undergo sterilization. This would effectively reduce the risk of introducing unwanted parasitoids.

- Wild-collected organisms are never shipped for SIT purposes. The product is mass-reared over many generations under quality control procedures aimed at eliminating unwanted organisms.
- The standard operating procedures for insect mass-rearing specifically provide mechanisms to prevent unwanted organisms.
- Biological control agents are sometimes shipped with live hosts or prey. Sterile insects are not.

For scenario 4, the consultants estimated that this risk would be many-fold less likely than the risk of introducing exotic organisms involved when moving biological control agents.

V. CONSEQUENCES IN CASE THE IDENTIFIED HAZARDS OCCURRED

Assuming that the identified hazards have occurred, the expert group described the following potential consequences:

Hazard 1: Outbreak of the target insect pest in a new area

The consequence of this hazard is the incursion or establishment of a serious insect plant pest. Negative impact of the new pest could include:

- Decrease in production of crops.
- Reduction in quality.
- Increase in production costs.
- Impact on trade.
- Impact on the environment.

These consequences apply to both incursions and establishment. In the case of incursions, the negative impact would be limited in scope and duration. This is because for an incursion, the conditions would not be suitable for permanent pest establishment (e.g. pest not able to survive winter or summer temperatures). However, in the event of pest establishment, eradication would be an option since SIT and other eradication tools are available for the species that are currently shipped as sterile insects.

Hazard 2: Increase of fitness of the local pest population through introduction of genetic material from the escaped insects.

The consequences of the existing local pest population could increase as a result of the introduction of new genetic material. This negative impact could be:

- Decreased production on already affected crops.
- Increased cost on already affected crops.
- Losses on other crop species.
- Environmental impact.
- Impact on trade.

With the existence of a local population, however, control practices may already be in place that will effectively manage the fitter pest. This may reduce the consequences.

Hazard 3: Unnecessary regulatory actions initiated due to failure to recognize the detected insect as sterile

This would apply only to pests subjected to an active surveillance programme. The detection and failure to recognize the insect as sterile could trigger several different actions:

- An increase in trapping (i.e. delimiting trapping) to assess the status of the detection.
- The initiation of an emergency programme for eradication.
- Disruption of internal movement and marketing by domestic regulatory actions.
- Prohibition of host product by a trading partner.

The implementation of these actions could have significant short-term financial implications.

Hazard 4: Introduction of exotic (new) contaminant organisms

The introduction of an exotic organism into a new ecosystem can have the following negative impacts:

- Direct damage on agricultural crops if the introduced organism is an exotic plant pest.
- Indirect damage on agricultural crops if introduced organism has a negative impact on beneficial organisms (pollinators, predators and parasites).
- Change in biodiversity and natural ecosystem.

This hazard is not unique to the shipment of sterile insects, and therefore should be considered in comparison to or in the context of the same hazard associated with shipments of other commodities, including non-biological shipments.

VI. ASSESSED RISK

Risk is the product of the likelihood of the hazard times the consequences. The potential consequences from the identified hazards could be significant. However, the extremely low likelihood of the hazards occurring indicates an overall negligible risk.

VII. CONCLUSIONS

The Consultants held detailed discussions and reviewed reference documents taking into consideration the scientific, technical and operational aspects of the Sterile Insect Technique (SIT) as applied to plant protection. Potential biological hazards and associated risks were identified for transboundary shipment of sterile insects for use in SIT programmes.

The consultants concluded the following:

A. Evidence indicates that SIT is likely to become more widely used. There is also a shift from government to private responsibility for certain aspects of the technology. This will require a more formal approach to activities involving more than one country. This is particularly relevant to production that results in transboundary shipments of the sterile insects.

B. The SIT has been used for nearly 50 years against insect pests of plants and animals. During this time, standard operating procedures have been developed by most individual programmes. In some cases, international standards have been developed

and are in use worldwide. For fruit fly species, the most important of these are the quality control and dosimetry manuals[1] (FAO/IAEA/USDA, 1998 and FAO/IAEA, 2000). The proper application of these manuals precludes the hazards identified by the Consultants Group from occurring.

C. There is a need for an internationally accepted code of conduct (or similar document) relating to transboundary shipments of sterile insects for use in SIT programmes. The International Plant Protection Convention (IPPC) is the international standard setting body for phytosanitary measures. Since the SIT is also used against insect pests of veterinary and medical importance, livestock insect pests and insect vectors of medical importance should be considered by the appropriate bodies in the near future.

D. The Consultants Group identified the hazards and assessed the risks associated with the transboundary shipment of sterile insects for SIT programmes. Both the likelihood and the consequences were considered for each of the hazards identified. A series of sequential events would be required for any of these potential hazards to occur. None of the events alone would constitute a hazard (refer to Figure 1).

E. The hazards identified, potential consequences and likelihood of the hazards occurring were:

1. Failure of sterilization, either total or partial, resulting in the target insect becoming an established pest in a new area, with the likelihood of 0.5×10^{-18}.
2. Introduction of new (intra-specific) genetic material into an established pest population by the "sterile insects", resulting in a more damaging insect pest, with the likelihood of 0.5×10^{-23}.
3. Failure to recognize a detected insect as sterile, resulting in an unnecessary and perhaps costly regulatory action, with the likelihood of 1×10^{-11}.
4. Introduction of an exotic contaminant organism, resulting in a new pest becoming established, was estimated to involve many folds less risk than from the movement of biological control agents, a risk already widely accepted.

F. Because of the sequence of events required for any of the above hazards to occur, the Consultants Group concluded that transboundary shipment would result in negligible risk with the use of FAO/IAEA operating procedures[4] regarding sterilization, handling/packaging and shipment of sterile insects.

VIII. RECOMMENDATIONS

The Consultants Group recommends that this discussion paper be sent to the IPPC Secretariat for consideration by the ICPM as the basis for a standard. The Group also recommend that this standard be separate from the International Standard for Phytosanitary Measures number 3 on biological control agents.

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[4] Comprehensive FAO/IAEA standard operating procedures exist for fruit fly species. For other plant pest species controlled by SIT, best practices are in place and standard procedures will be harmonized internationally over time. The Consultants Group believes that the risk will be negligible from transboundary shipment of these other species as well, when best practices are applied.

Furthermore, the consultants recommend that the appropriate international bodies should assess the risks from transboundary shipment of insect pests of livestock and insects of medical importance controlled through SIT, and develop harmonized guidance.

IX. REFERENCES

Relevant guidelines for SIT

American Society for Testing and Materials (ASTM) (1999), Standard Guide for Irradiation of Insects for Sterile Release Programs. Designation: ASTM E 1940 - 98. 11 pp.

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USDA/APHIS (1999) Medfly Cooperative Eradication Program, Lake Forest California, Environmental Assessment. 12 pages.

USDA/APHIS (1999) Fruit Fly Cooperative Control Program. Draft Environmental Impact Statement. 356 pages.

Appendix E: History of Transboundary Shipments of Sterile Tephritid Fruit Flies (1963-2015)

Year	Tephritid species	Site of production	Amount shipped *(million pupae)*	Recipient	Observations
1963-1990	Mexican fruit fly, *Anastrepha ludens*	Monterrey, Mexico	Unknown	Texas, USA	
1970/71	Mediterranean fruit fly, *Ceratitis capitata*	Seibersdorf, Austria	Unknown	Procida, Italy, and Greece	Relatively small amount since sterile flies were used for field trials
1970	Mediterranean fruit fly	Costa Rica	Unknown	Nicaragua	Relatively small amount since sterile flies were used for field trials
1975-1977	Mediterranean fruit fly	Madrid, Spain	302	Canary Islands	
1978	Mediterranean fruit fly	Seibersdorf, Austria	Unknown	Guatemala	Sterile pupae shipped from the IAEA laboratories (Seibersdorf) to a packing and emergence facility in Guatemala for field trials and staff training in SIT techniques
1979-2000	Mediterranean fruit fly	Chiapas, Mexico	280,000	Guatemala	Biweekly transboundary shipments have been carried out for the past 21 years
1989-1994	Mediterranean fruit fly	Chiapas, Mexico	6,670	California, USA	To assist the CDFA in eradication of Mediterranean fruit fly outbreaks
1990	Mediterranean fruit fly	Chiapas, Mexico	552	Chile	Sterile flies donated by the Mexican government to the eradication project in Arica, Chile

Year	Tephritid species	Site of production	Amount shipped (million pupae)	Recipient	Observations
1992-1993	Mexican fruit fly	Mission, Texas	3500	State of Baja California Norte, Baja California Sur, and Sonora, Mexico	Eradication programme.
1994-1996	Mexican fruit fly	Tapachula, Chiapas, Mexico	11000	State of Baja California Norte, Sonora, Coahuila and Sinaloa Mexico	Eradication programme
1997-2000	West Indian fruit fly, A. obliqua	Tapachula, Chiapas, Mexico	3900	State of Sinaloa and Tamaulipas	Eradication Programme
1989-1990	Mediterranean fruit fly	Seibersdorf, Austria	Unknown	Israel	Pilot trials
1994	Mediterranean fruit fly	Seibersdorf, Austria	60	Tunisia	Pilot trials
1996-2000	Mexican fruit fly	Chiapas, Mexico	2,511	California, USA	To suppres/Eliminate Mexican fruit fly outbreaks
1994-2001	Mediterranean fruit fly	El Pino, Guatemala	51,800	California, USA	To assist the CDFA in eradication of Mediterranean fruit fly outbreaks
1997/98	Mediterranean fruit fly	Madeira, Portugal	206	Israel	In support of pilot suppression programme
1997-2000	Mediterranean fruit fly	El Pino, Guatemala	1,000	Israel	In support of pilot suppression programme
1998-2001	Mediterranean fruit fly	El Pino, Guatemala	19,500	Florida, USA	To assist the State of Florida in eradication of Mediterranean fruit fly outbreaks
1999-2000	Mediterranean fruit fly	El Pino, Guatemala	600	South Africa	In support of pilot suppression programme
2007-2013	Mediterraneanfruit fly	Bio-Fly, Israel	504	Jordan	In support of pilot suppression programme
2009-2011	Mediterranean fruit fly	Madeira, Portugal	696	Morocco	In support of pilot suppression programme

Year	Tephritid species	Site of production	Amount shipped (million pupae)	Recipient	Observations
2010, 2012, and 2014	Mediterranean fruit fly	Bio-Fly, Israel	969	Croatia	In support of pilot suppression programme
2011 and 2013	Mediterranean fruit fly	Valencia, Spain	425	Croatia	In support of pilot suppression programme