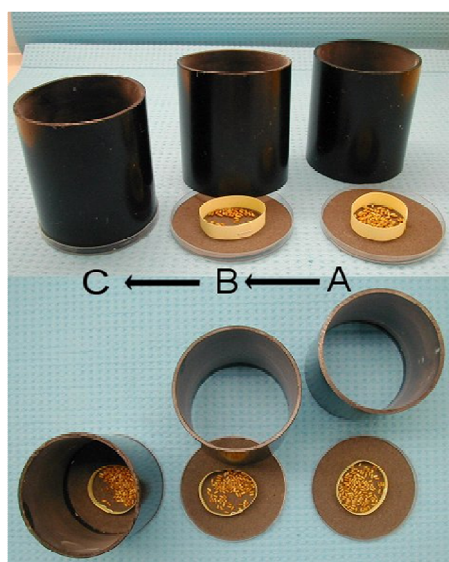


Product Quality Control for Sterile Mass-Reared and Released Tephritid Fruit Flies



Version 6.0

May 2014

IAEA, Vienna, Austria

Preamble to Version 6.0

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 demonstrating 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:

- 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 and 2010. 7th and 8th 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.

DRAFT

The proper citation for this document is:

FAO/IAEA/USDA. 2014. Product Quality Control for Sterile Mass-Reared and Released Tephritid Fruit Flies, Version 6.0. *International Atomic Energy Agency*, Vienna, Austria. 164 pp.

Table of Contents

1. Introduction	7
1.1. Scope.....	7
1.2. Background	10
1.3. Rationale	10
1.3.1. The Need for Product Quality Control in SIT Programmes	10
1.3.2. Mating Behaviour and Sterile Male Competitiveness in the Field	11
1.3.3. Changes from the Earlier Manuals.....	12
1.3.4. Management of Product Quality Control.....	13
1.3.5. Future Plans to Update this Manual	13
1.4. Data Analysis, Presentation and Communication	14
1.5. General Comments	15
1.6. Acknowledgements	15
1.7. Relevant Literature.....	16
2. Required Routine Quality Control Tests at Mass-Rearing Facilities	19
2.1. Guidelines for Sampling Insects for Routine QC Tests.....	19
2.2. Quality Control Tests after Mass-Rearing (pre-irradiation)	21
2.2.1. Pupal Weight.....	21
2.2.2. Emergence and Flight Ability	23
2.2.3. Survival under Stress	27
2.3. Quality Control Tests Post-irradiation	29
2.3.1. Radiation Indicator.....	29
2.3.2. Emergence and Flight Ability	29
2.3.3. Survival under Stress	29
2.3.4. Sex Ratio and Timing of Emergence.....	29
2.4. Relevant Literature.....	31
3. Irradiation	33
3.1. Development Stage/Age of Insects.....	33
3.2. Pre-irradiation Procedures.....	34
3.3. Irradiation and Process Control.....	37
3.4. Relevant Literature.....	40
4. Dosimetry	42
4.1. Dosimetry System	43
4.2. Establishing Traceability	48
4.3. Calibration of Gafchromic® Dosimetry System.....	52
4.4. Uncertainty.....	55
4.5. Relevant Literature.....	57
5. Packing and Shipping Procedures	59
5.1. Packaging Procedures.....	59
5.2. Shipping and Handling Procedures	62

5.3. Shipping Documents	63
5.4. Relevant Literature.....	64
6. Routine quality control tests at fly emergence and release facilities.....	65
6.1. Guidelines for Sampling Insects for Routine QC Tests.....	65
6.2. Quality Control Tests on Arrival	65
6.2.1. Radiation indicator	66
6.2.2. Pupal weight	66
6.2.3. Emergence and flight ability	67
6.2.4. Survival under stress	68
6.2.5. Sex Ratio and Timing of Emergence.....	68
6.3. Quality Control Post Handling/Chilling	69
6.3.1. Absolute fliers	69
6.3.2. Survival chilled/released flies under stress	71
6.4. Quality Control Post Release.....	72
6.4.1. Absolute fliers	72
6.4.2. Survival chilled/released flies under stress	73
6.5. Relevant Literature.....	74
7. Required periodic quality control at the mass-rearing and/or fly emergence and release facilities.....	75
7.1. Sterility Test	75
7.2. Mating Performance Field Cage Test	77
7.3. Survival in the Field.....	93
7.4. Fly Irritability Test	94
7.5. Release-Recapture Test of Dispersal and Survival	97
7.6. Relevant Literature.....	100
8. Ancillary tests.....	106
8.1. Activity Test	106
8.2. Detailed Lekking and Courtship Behaviour Observations	107
8.3. Pheromone Compatibility Test.....	109
8.4. Male Frequency of Mating Test	111
8.5. Female Propensity to Remate Test.....	113
8.6. The Fried Test	114
8.7. Open Field Fried Test	117
8.8. Survival in Field Cages	118
8.9. Pupal Size	120
8.10. Relevant literature	122
9. Forms for Recording Quality Control Data	124
9.1. Pupal Weight Assessment Form.....	124
9.2. Emergence and Flight Ability Assessment Form.....	125
9.3. Survival under Stress Assessment Form.....	126
9.4. Sex Ratio and Timing of Emergence Assessment Form	127
9.5. Datasheet for Shipment of Sterile Pupae	129

9.6. Sterility Assessment Form	130
9.7. Mating Performance Assessment Form	131
9.8. Graphic Representation of Indices of Mating Performance	132
9.9. Fly Irritability Assessment Form	133
Appendix A: Chronology of Product Quality Control of Tephritid Flies for Use in SIT Programmes.....	134
Appendix B: Known Sources of Key Equipment and Supplies.....	138
Appendix C: Terminology.....	141
Appendix D: Transboundary Shipments of Sterile Insects	146
Appendix E: History of Transboundary Shipments of Sterile Tephritid Fruit Flies (1963-2013)	162

DRAFT

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 new addition in this version is 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 (**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 cause or 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 handling and shipping.

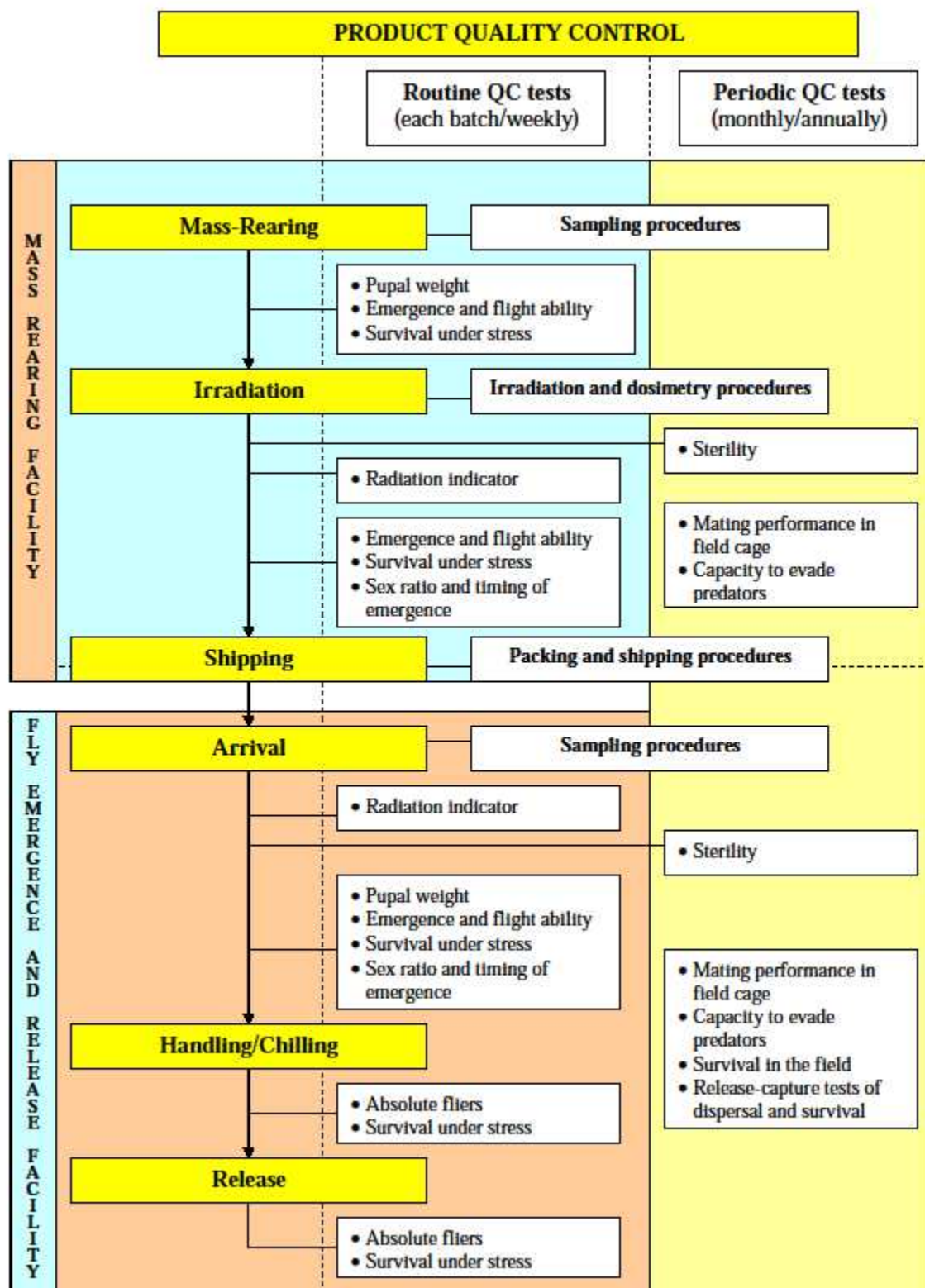


Figure 1.1: Flowchart of the product quality control for sterile mass-reared and released tephritid flies.

Additional product QC tests have been developed and their use is optional (see Chapter 8 Ancillary tests). *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). This revision adds standards for new species and updates previous threshold values as a result of the increasing use of the SIT. 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 chemical controls.

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. Correlations could be made in some instances, but not all. 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 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. Mass-rearing and releasing a given number of sterile fruit flies into the field is only part of a successful SIT programme.

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 “lek” mating systems. First, the behaviour performed by the male in these mating systems is unusually 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 also are attracted by the pheromone and, from the sum of physical and chemical factors, 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 approaches a male, he begins his courtship ritual of head-rocking and pulsed wing fanning. Communication to the female 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 (and does so about half the time) 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 and do effect phenotypic 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) suggests that lapses in mating compatibility are 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 et 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

Establishing specifications for the quality of insects to be used in an SIT programme is only the first step. Specifications have little value if the user cannot expect that they will be routinely met. That expectation requires an adequate QC programme at the production facility; a second requirement is analysis, verification, and communication between producer and user. 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 measurements of each parameter will be accumulated during a period when the rearing process is thought to be “under control” (i.e., when fly quality is, subjectively, “good”). 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 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* and *Bactrocera* mass-rearing, fly emergence and release facilities and/or research laboratories in Argentina, Australia, Austria, Chile, Croatia, Greece, Guatemala, Israel, Japan, Mexico, Peru, Philippines, Portugal, Spain and the United States (California, Florida, Hawaii, Texas).

Upgrades for Version 6 of this manual are based on a Consultants Meeting held in Vienna during October 2010 with fruit fly quality control experts from Argentina, Australia, Guatemala, Mexico and the USA.

1.7. Relevant Literature

- Agee, H. R. and D. L. Chambers. 1980. Fruit fly quality monitoring. Nat. Inst. Agric. Sci. 7-15.
- Annual book of ASTM standards, Vol. 12.02 American Society for Testing and Materials, Philadelphia, PA, USA.
- Bloem, S., K. A. Bloem and A. L. Knight. 1998. Assessing the quality of mass-reared codling moths (Lepidoptera: Tortricidae) by using field release-recapture tests. J. Econ. Entomol. 91: 1122-1130.
- Boller, E. F. and C. O. Calkins. 1984. Measuring, monitoring and improving the quality of mass-reared Mediterranean fruit flies, *Ceratitis capitata* (Wiedmann) 3. Improvement of quality by selection. Z. angew. Entomol. 98: 1-15.
- Boller, E.F. and D.L. Chambers. 1977. Quality control: an idea book for fruit fly workers. IOBC/WPRS Bull. 1977/5. 162 pp.
- Boller, E. F., U. Remund, B. I. Katsoyannos and W. Berchtold. 1977. Quality control in European cherry fruit fly: evaluation of mating activity in laboratory and field-cage tests. Z. angew. Entomol. 83: 183-201.
- Boller, E. F., B. I. Katsoyannos, U. Remund and D. L. Chambers. 1981. Measuring, monitoring and improving the quality of mass-reared Mediterranean fruit flies, *Ceratitis capitata* Wiedmann. Z. angew. Entomol. 92: 67-83.
- Brazzel, J.R., C. Calkins, D.L. Chambers and D.B. Gates. 1986. Required quality control tests, quality specifications, and shipping procedures for laboratory produced Mediterranean fruit flies for sterile insect control programs. APHIS 81-51, USDA-APHIS, Hyattsville, MD, USA.
- Calkins, C.O., E.F. Boller, D.L. Chambers and Y. Ito. 1979. Quality control in *Ceratitis capitata*: a training manual for the international course on quality control held in Castellon, Spain, 17-27 September, 1979.
- Calkins, C.O., K. Bloem, S. Bloem and D. L. Chambers. 1994. Advances in measuring quality and assuring good field performance in mass reared fruit flies. 85-96. In Calkins, C.O., W. Klassen, and P. Liedo [eds.], Fruit Flies and the Sterile Insect Technique. CRC Press, Boca Raton, Florida, USA.
- Calkins, C.O., T.R. Ashley and D. L. Chambers. 1996. Implementation of technical and managerial systems for quality control in Mediterranean fruit fly (*Ceratitis capitata*) sterile release programs. 399-404. In McPherson, B.A. and G.C. Steck [eds.], Fruit fly pests: a world assessment of their biology and management. St. Lucie Press, Delray Beach, Florida, USA.
- Chambers, D.L. 1975. Quality in Mass-Produced Insects: Definition and evaluation. pp.19-32. In Controlling Fruit Flies by the Sterile-Insect Technique - Panel Proceedings Series. International Atomic Energy Agency, Vienna, Austria, STI/Pub/392
- Chambers, D. L. 1977. Quality control in mass rearing. Ann. Rev. Entomol. 22: 289-308.
- Chambers, D. L. 1980. Review SIRM with special reference to quality control. 1-5. In Proceedings of a Symposium on Fruit Fly Problems. Kyoto & Naha, Japan. August 9-12, 1980. National Institute of Agricultural Services. Yatabe, Ibaraki, Japan.
- Chambers, D.L., C.O. Calkins, E.F. Boller, Y. Ito and R.T. Cunningham. 1983. Measuring, monitoring, and improving the quality of mass-reared Mediterranean fruit flies, *Ceratitis capitata* Wied. 2. Field tests for confirming and extending laboratory results. Z. angew. Entomol. 95: 285-303.
- Crystal, M. M. and E. Ramirez. 1975. Screwworm flies for sterile-male release: laboratory tests of the quality of candidate strains. J. Med. Entomol. 12: 418-422.

- Crystal, M. M. and C. J. Whitten. 1976.** Screwworm flies for sterile-male releases: laboratory observations of the quality of newer candidate strains. *Ann. Entomol. Soc. Am.* 69: 621-624.
- Dame, D. A. 1989.** The relationship of research to total quality control with special reference to sterile insect technique. *J. Appl. Ent.* 108: 476-482.
- FAO. 2007.** Guidance for packing, shipping, holding and release of sterile flies in area-wide control programmes. *FAO Plant Production & Protection Paper 190*, ed. Walter Enkerlin. pp.134.
- FAO/IAEA. 2004** Coordinated Research Project: Quality Assurance of Mass Produced and Released Fruit Flies.
- FAO/IAEA. 2009.** Coordinated Research Project: Development of Mass Rearing for New World (*Anastrepha*) and Asian (*Bactrocera*) Fruit Flies.
- FAO/IAEA. 2009.** Coordinated Research Project: Improving Sterile Male Performance in Fruit Fly SIT programmes.
- Florida Entomologist. 2007.** Vol 90 (1): 1-179. Includes 24 of 30 papers that comprise the Proceedings of a 5-year FAO/IAEA Coordinated Research Project on Fruit Fly Quality, Control, and Behavior. Edited by C. Cáceres, J. Hendrichs, E. B. Jang, D. O. McInnis, A. S., Robinson, and T. E. Shelly.
- Programa Regional Moscamed. 2002.** Manual de Control Autocida de la mosca del Mediterraneo esteril por el sistema de adulto frio. 63 pp.
- Hibino, Y. and O. Iwahashi. 1991.** Appearance of Wild Females Unreceptive to Sterilized Males on Okinawa Is. in the Eradication Program of the Melon Fly, *Dacus cucurbitae* Coq. (Diptera: Tephritidae). *Appl. Entomol. Zool.* 26 (2): 265-270.
- Hibino, Y. and O. Iwahashi. 1989.** Mating Receptivity of Wild Type Females for Wild Type Males and Mass-Reared Males in the Melon Fly, *Dacus cucurbitae* Coq. (Diptera: Tephritidae). *Appl. Entomol. Zool.* 24 (1): 152-154.
- Huettel, M.D. 1976.** Monitoring the quality of laboratory-reared insects: a biological and behavioral perspective. *Environ. Entomol.* 5: 807-814.
- Klee, A. 1981.** International meeting on *Ceratitis capitata* quality control. Guatemala, October, 1981.
- Koyama, J. 1982.** Quality problems in the mass-rearing for the melon fly, *Dacus cucurbitae* coquillett. *JARQ.* 16: 181-187.
- Leppla, N.C. and T. R. Ashley. 1989.** Quality control in insect mass production: a review and model. *Bull. Entomol. Soc. Am.* 23: 33-43.
- Mastangelo T., A.G. Parker, A. Jessup, R. Pereira, D. Orozco-Davila, A. Islam, T. Dammalage and J.M.M. Walder. 2010.** A new generation of X ray irradiators for insect sterilization. *J. Econ. Entomol.* 103(1): 85-94.
- McInnis, D.O., D.R. Lance and C.G. Jackson. 1996.** Behavioral resistance to the sterile insect technique by the Mediterranean fruit fly (Diptera: Tephritidae) in Hawaii. *Ann. Entomol. Soc. Am.* 89(5): 739-744.
- Meats, A. 1998.** A quality assurance measure for field survival rates of released sterile flies based on recapture rates. *Gen. Appl. Entomol.* 28: 39-46.
- Miyatake T. and M. Yamagushi. 1993.** Active quality control in mass reared melon flies: quantitative genetic aspects. In: *Management of Insect Pests: Nuclear and Related Molecular Genetic Techniques*, pp. 201-213. IAEA Vienna.
- Orozco, D.O., A.G. Schwarz and A. Perez Romero. 1983.** Manual de procedimientos de control de calidad. Dirección General de Sanidad Vegetal, Secretaría de Agricultura y Recursos Hidraulicos. Talleres Graficos de Nacion. Mexico, D.F., 137pp.

Prokopy, R.J., and J. Hendrichs. 1979. Mating behavior of *Ceratitis capitata* on a field-caged host tree. *Ann. Entomol. Soc. Am.* 72: 642-648.

Santiago Martínez, G., Orozco Dávila, D., Hernández Ibarra, M., Luis Álvarez, J.H. y Bravo López, J.J. 2012. Manual de Control de Calidad de Moscas Estériles: Procedimientos para evaluar el producto en centros de empaque. Dirección de Moscas de la Fruta, DGSV-SENASICA, SAGARPA, Mexico, D.F. 34 pp. <http://www.senasica.gob.mx/?doc=10106>.

Serghiou, C.S. 1977. Selected factors affecting the quality of Mediterranean fruit fly used in sterile release programs. *J. Econ. Entomol.* 70: 351-356.

Smith, P.H., C.A. Konovalov, G.G. Foster and R.W. Kerr. 1981. Assessment of the quality of mass-reared *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) males treated with dieldrin as larvae in a female-killing procedure. *Bull. Entomol. Res.* 71: 1-10.

Southwood, T.R.E. 1978. Ecological methods with particular reference to the study of insect populations (2nd Ed.). Wiley & Sons, N.Y., USA.

SPSS. 1996. SYSTAT 6.0 for Windows: Graphics. SPSS, Inc., Chicago, IL, USA.

Stock, M.W. and J.L. Robertson. 1982. Quality assessment and control in a western spruce budworm laboratory colony. *Entomol. Exp. Appl.* 32: 28-32.

Tsiropoulos, G.J. 1980. Quality problems associated with the mass-production of *Dacus oleae* (Gmel.) (Dipt., Trypetidae). *Z. angew. Entomol.* 90: 413-420.

Tsiropoulos, G.J. and A.G. Manoukas. 1977. Adult quality of *Dacus oleae* affected by larval crowding and pupal irradiation. *Ann. Entomol. Soc. Am.* 70: 916-918.

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

Vreysen M.J.B., A.S. Robinson and J. Hendrichs (eds.). 2007. Area-Wide Control Insect Pests: From Research to Field Implementation. 792 p.

Walker, M.L., W.L. McLaughlin, J.M. Puhl and P.J. Gomes. 1997. Radiation-field mapping of insect irradiation canisters. *Appl. Radiat. Isot.* 48(1): 117-125.

Zavala, J.L., M.M. Fierro, A.J. Schwarz, D.H. Orozco and M. Guerra. 1985. Dosimetry practice for the irradiation of the Mediterranean fruit fly *Ceratitis capitata* (Wied.). 23-30. In IAEA [ed.] High dose dosimetry, Proceedings of the International Symposium, IAEA STI/PUB/671, IAEA, Vienna, Austria.

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 normal rearing conditions the quality of insects produced by a facility will differ from batch to batch due to variation 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. Even in individual groups of mass-reared tephritids, quality can vary within and among trays or other rearing units. Such known sources of variation in insect quality need to be accounted for when sampling insects for QC testing so that the data from the tests estimate with some precision the size and quality of the flies produced.

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, random samples are taken from across the production for the day (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 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 $\approx 0.1/1.96$ (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 $SE = s/\sqrt{n}$, 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 precise estimates from fewer samples. It is recommended that individual facilities develop their own sampling protocols which give acceptable precision for the least number of samples to be processed, based on their own data. This approach also leaves more pupae available for irradiation and shipment.

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 a valuable indicator of overall viability of pupae and correlates with size of the resulting adult flies. Evidence shows that larger 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.

Historically, two methods have been used to measure pupal size: weight and diameter. Both measures are influenced by the effect of water loss from the pupae as they age. Unequal rates of water loss from differences in humidity or temperature conditions in different rearing facilities result also in different relationships between pupal weight and adult fly size.

Whether weight or diameter is used depends largely on the agreement between the producer and the user of the sterile insects, although most facilities have adopted pupal weight as the measure of size for product quality control. It is also the easier of the two measurements to perform. However, as both methods would be valuable for process quality control (e.g. sorting pupae by diameter gives better information on size distribution) both are described. 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.

As the effects of washing on size measurements have not been evaluated adequately, pupae should not be washed before measuring size for product quality control purposes. Instead all visible trash, such as vermiculite and sawdust should be removed from the sample, along with any pupae with trash attached.

Test Frequency: Pupal size, particularly 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.

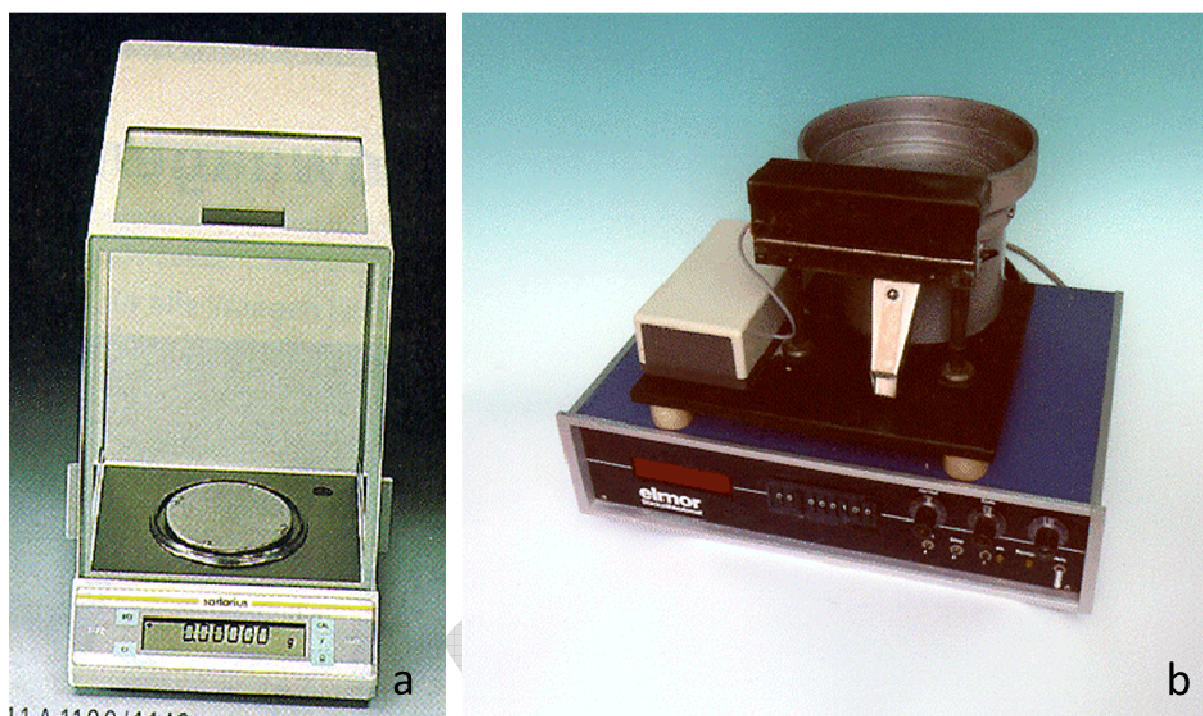


Figure 2.1: (a) A typical balance used to weigh pupae; (b) Automated pupal counter to count pupae in samples.

Procedure

Mean pupal weight is determined by taking samples two days before emergence (i.e. on the day when pupae are typically irradiated and shipped) 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
<i>Ceratitis capitata</i>		
bisexual strain	7.00	7.50
genetic sexing strain (tsl)	7.50	7.80
<i>Anastrepha ludens</i>	17.08	17.71
<i>Anastrepha obliqua</i>	13.52	14.22
<i>Anastrepha suspensa</i>	10.00	14.00
<i>Bactrocera cucurbitae</i>	13.00	13.50
<i>Bactrocera dorsalis</i>	12.30	12.90
<i>Bactrocera oleae</i>	6.00	6.54
<i>Bactrocera tryoni</i>	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, inappropriate holding conditions during pupal maturation, or other factors that could reduce the viability of the released insects. As small size 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 produce flies 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.

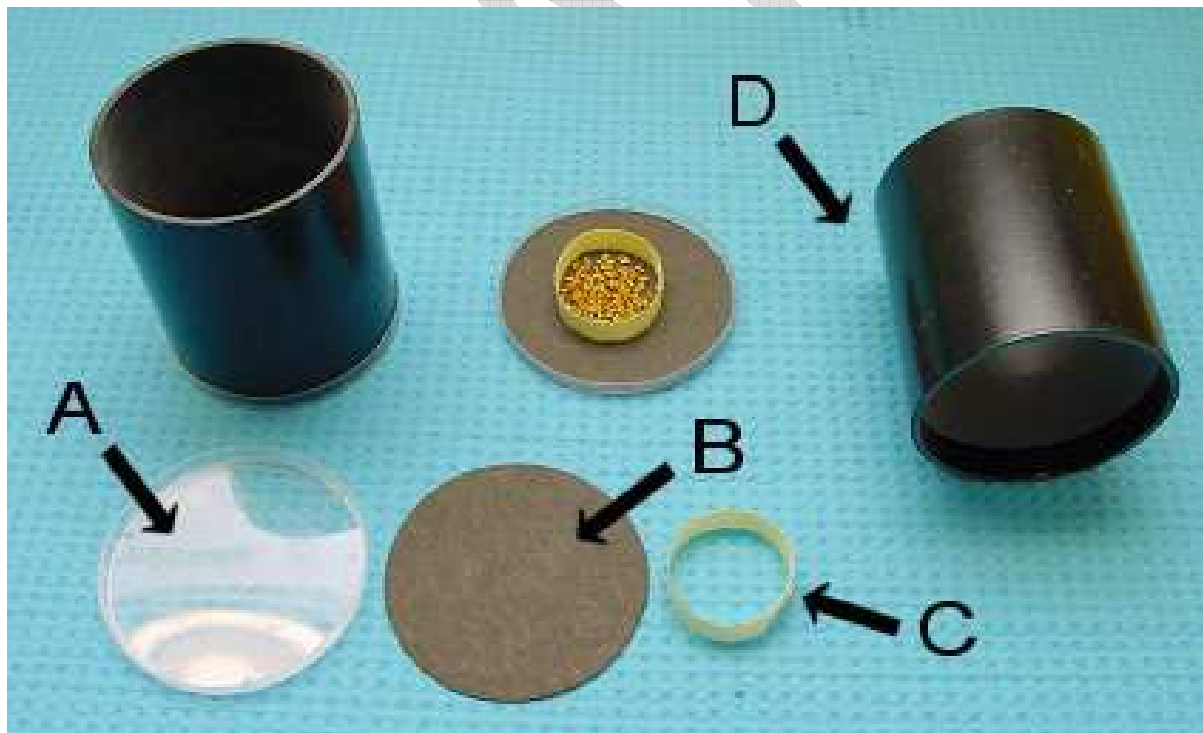


Figure 2.2: Equipment for the emergence and flight ability test.

Test Conditions

- Temperature $25 \pm 1^\circ \text{C}$
- Humidity $65 \pm 15\% \text{ RH}$
- 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**.

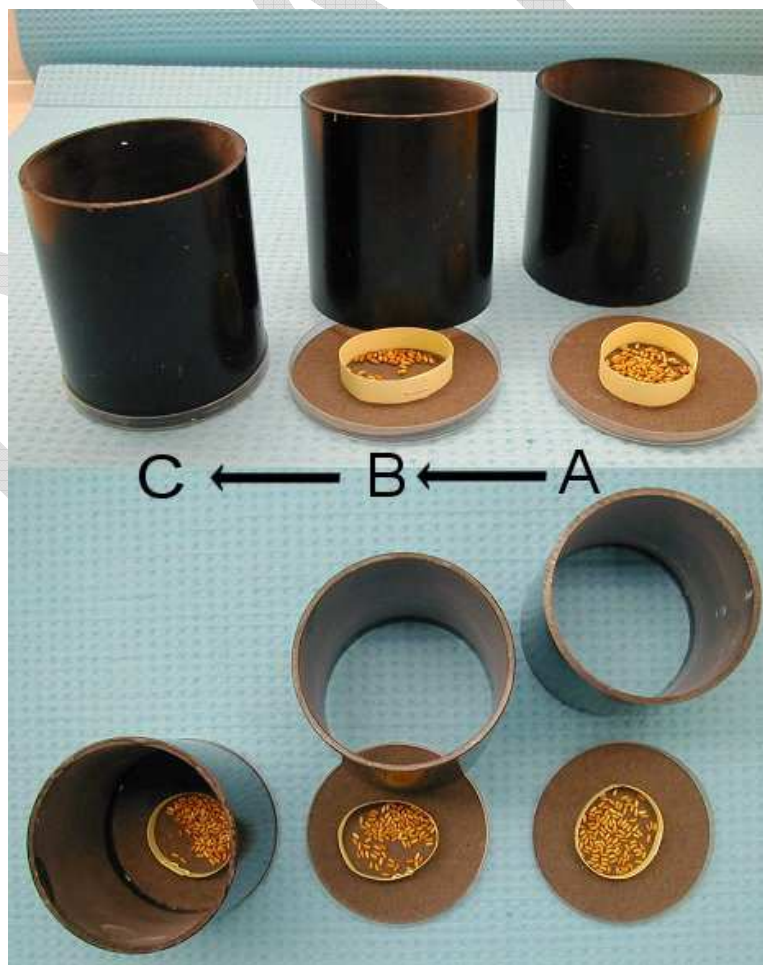


Figure 2.3: Setting up the emergence and flight ability test.

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 (%)						
<i>Ceratitis capitata</i>						
bisexual strain	85	80	75	93	90	88
genetic sexing strain (tsl)	75	70	70	80	75	70
<i>Anastrepha ludens</i>	91	90	80	93	92	86
<i>Anastrepha obliqua</i>	92	90	86	94	92	89
<i>Anastrepha suspensa</i>	85	80	75	93	90	88
<i>Bactrocera cucurbitae</i>	90	80	70	92	90	85
<i>Bactrocera dorsalis</i>	82	74	70	90	79	75
<i>Bactrocera oleae</i>	80	75	55	85	80	60
<i>Bactrocera tryoni</i>	80	70	65	85	80	75
Species/strain	Minimum			Acceptable mean		
	Pre-irradiation	Post-irradiation	Post - shipment	Pre-irradiation	Post-irradiation	Post - shipment
Fliers (%)						
<i>Ceratitis capitata</i>						
bisexual strain	75	70	65	85	82	78
genetic sexing strain (tsl)	70	65	65	75	70	65
<i>Anastrepha ludens</i>	88	86	71	90	89	79
<i>Anastrepha obliqua</i>	89	85	77	90	88	80
<i>Anastrepha suspensa</i>	75	70	65	85	80	75
<i>Bactrocera cucurbitae</i>	80	75	-	85	83	-
<i>Bactrocera dorsalis</i>	75	69	62	83	77	72
<i>Bactrocera oleae</i>	70	65	50	77	72	55
<i>Bactrocera tryoni</i>	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. A high degree of variability or consistent downward trend can indicate an emerging problem that requires corrective action in rearing or handling 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 pupae. For comparative purposes pupae 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/cm²) 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 ± 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 6.2.3). 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 interval (h)	Minimum survival (%)
<i>Ceratitis capitata</i>		
bisexual strain	48	50
genetic sexing strain (tsl)	48	65
<i>Anastrepha ludens</i>	72	55
<i>Anastrepha obliqua</i>	48	40
<i>Anastrepha suspensa</i>	48	n/a
<i>Bactrocera cucurbitae</i>	n/a	n/a
<i>Bactrocera dorsalis</i>	n/a	n/a
<i>Bactrocera oleae</i>	n/a	n/a
<i>Bactrocera tryoni</i>	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 a relative measure of the stored nutrients (food reserves) and water found in the lab 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 2.6**).

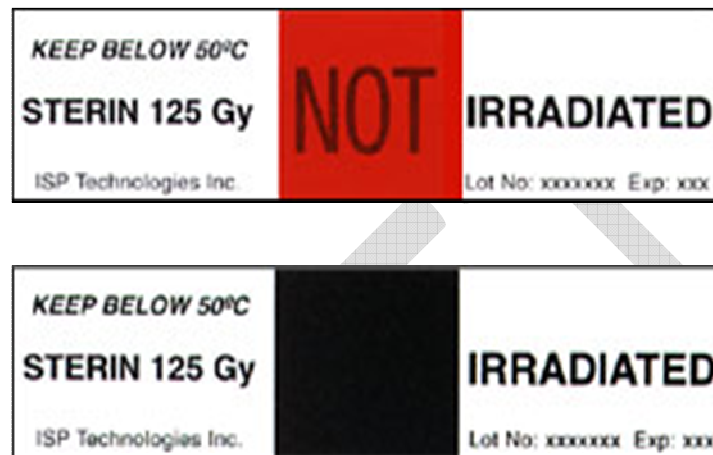


Figure 2.4: Radiation sensitive indicators before (top) and after (bottom) exposure at dose > 125 Gy (© ISP 2002).

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. Inappropriate temperatures during rearing of genetic sexing strains may also distort the sex ratio.

Equipment

- Screened emergence grids with 100 individual cells. These are constructed from the open plastic grids that are often used under fluorescent lights. 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 genetic sexing strains should be over 99% male. 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

Annual book of ASTM standards, Vol. 12.02 American Society for Testing and Materials, Philadelphia, PA. USA.

Bloem, K., S. Bloem, D. Chambers and E. Muñiz. 1993. Field evaluation of quality: release-recapture of sterile medflies of different sizes. 295-296. *In* Aluja, M and P. Liedo [eds.] *Fruit flies: biology and management*. Springer-Verlag, New York, NY, USA.

Boller, E.F. and D.L. Chambers. 1977. Quality control: an idea book for fruit fly workers. IOBC/WPRS Bull. 1977/5. 162 p.

Boller, E.F., B.I. Katsoyannos, U. Remund and D.L. Chambers. 1981. Measuring, monitoring, and improving the quality of mass-reared Mediterranean fruit flies, *Ceratitis capitata* Wied. 1. The RAPID quality control system for early warning. *Z. angew. Entomol.* 92: 67-83.

Brazzel, J.R., C. Calkins, D.L. Chambers and D.B. Gates. 1986. Required quality control tests, quality specifications, and shipping procedures for laboratory produced Mediterranean fruit flies for sterile insect control programs. APHIS 81-51, USDA-APHIS, Hyattsville, MD.

Burk, T. and J.C. Webb. 1983. Effect of male size on calling propensity, song parameters, and mating success in Caribbean fruit flies, *Anastrepha suspensa* (Loew) (Diptera: Tephritidae). *Ann. Entomol. Soc. Am.* 76: 678.

Caceres 2002 Mass rearing of temperature sensitive genetic sexing strains in the Mediterranean fruit fly *Ceratitis capitata*. *Genetica* 116: 107-116.

Calkins, C.O., and T.R. Ashley. 1989. The impact of poor quality mass-reared Mediterranean fruit flies on the sterile insect technique used for eradication. *J. Appl. Ent.*, 108:401-408.

Calkins, C.O., T.R. Ashley and D.L. Chambers. 1996. Implementation of technical and managerial systems for quality control in Mediterranean fruit fly (*Ceratitis capitata*) sterile release programs. 399-404. *In* McPherson, B.A., and G.C. Steck [eds.], *Fruit fly pests: a world assessment of their biology and management*. St. Lucie Press, Delray Beach, Florida, USA.

Calkins, C.O., K. Bloem, S. Bloem and D.L. Chambers. 1994. Advances in measuring quality and assuring good field performance in mass reared fruit flies. 85-96. *In* Calkins, C.O., W. Klassen, and P. Liedo [eds.], *Fruit Flies and the Sterile Insect Technique*. CRC Press, Boca Raton, Florida, USA.

Calkins, C.O., E.F. Boller, D.L. Chambers and Y. Ito. 1979. Quality control in *Ceratitis capitata*: a training manual for the international course on quality control held in Castellon, Spain, 17-27 September, 1979.

Calkins, C.O. and A.G. Parker. 2005. Sterile Insect Quality. 269-296. *In* Dyck, V.A., J. Hendrichs, and A.S. Robinson [eds.]. *Sterile Insect Technique: Principles and Practice in Area-Wide Pest Management*. Springer, Dordrecht, The Netherlands.

Chambers, D.L. 1980. Review SIRM with special reference to quality control. 1-5. *In* Proceedings of a Symposium on Fruit Fly Problems. Kyoto & Naha, Japan. August 9-12, 1980. National Institute of Agricultural Services. Yatabe, Ibaraki, Japan.

Chambers, D.L., C.O. Calkins, E.F. Boller, Y. Ito and R.T. Cunningham. 1983. Measuring, monitoring, and improving the quality of mass-reared Mediterranean fruit flies, *Ceratitis capitata* Wied. 2. Field tests for confirming and extending laboratory results. *Z. angew. Entomol.* 95: 285-303.

- Churchill-Stanland, C., R. Stanland, T.T.Y. Wong, N. Tanaka, D.O. McInnis and R.V. Dowell. 1986.** Size as a factor in the mating propensity of Mediterranean fruit flies, (*Ceratitidis capitata* (Diptera: Tephritidae), in the laboratory. J. Econ. Entomol. 79: 614-619.
- Collins, S.R. and Taylor, P.W. 2010.** Flight ability procedures for mass-reared Queensland fruit flies, *Bactrocera tryoni*: an assessment of some variations. Ent. exp. appl. 136: 308-311.
- Hernández, E., S. Flores Breceda, M.L. Sosa Iturbe and H. Ezquivel 2005.** Tamaño de unidad muestral y número de repeticiones para la estimación de los parámetros de desarrollo de *Anastrepha obliqua* y *A. ludens* (Diptera: Tephritidae). Folia Entomol. Mex., 44: 155-164.
- Klee, A. 1981.** International meeting on *Ceratitidis capitata* quality control. Guatemala, October, 1981.
- Krainacker, D.A., J.R. Carey and R.I. Vargas. 1989.** Size-specific survival and fecundity for laboratory strains of two tephritid (Diptera: Tephritidae) species: implications for mass rearing. J. Econ. Entomol. 82: 104.
- Leppla, N.C. 2009** The basics of quality control for insect rearing. 289-306. In J.C. Schneider [ed.]. Principles and Procedures for Rearing High Quality Insects. Mississippi State University.
- Orozco, D. and R.O. Lopez. 1993.** Mating competitiveness of wild and laboratory mass-reared medflies: effect of male size. 185-188. In Aluja, M., and P. Liedo [eds.,] Fruit flies: biology and management. Springer-Verlag, New York, NY, USA.
- Orozco, D.O., A.G. Schwarz and A. Perez Romero. 1983.** Manual de procedimientos de control de calidad. Dirección General de Sanidad Vegetal, Secretaría de Agricultura y Recursos Hidraulicos. Talleres Graficos de Nacion. Mexico, D.F. p 137.
- Ruhm, M.E. and C.O. Calkins. 1981.** Eye-color changes in the Mediterranean fruit fly *Ceratitidis capitata* pupae, a technique to determine pupal development. Ent. exp. appl. 29: 237-240.
- Southwood, T.R.E. and P.A. Henderson 2000.** Ecological methods (3rd Ed.). Blackwell Science, Oxford, UK.
- Wajnberg, E. 2003.** Basic statistical methods for quality control workers. 305-314 In J.C. van Lenteren [ed.] Quality Control and Production of Biological Control Agents. Theory and Testing Procedures. CABI Publishing, Cambridge, MA, USA.

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
- Intel QX5 computer microscope (2007 Digital Blue, Inc., Microsoft Corporation, USA)

Procedure

The pupae are first dissected to observe changes in pupal eye colour pupae. The shell of the anterior part of the puparium is carefully removed to expose the eyes of the developing imago to be photographed. 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 evolution of *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**.

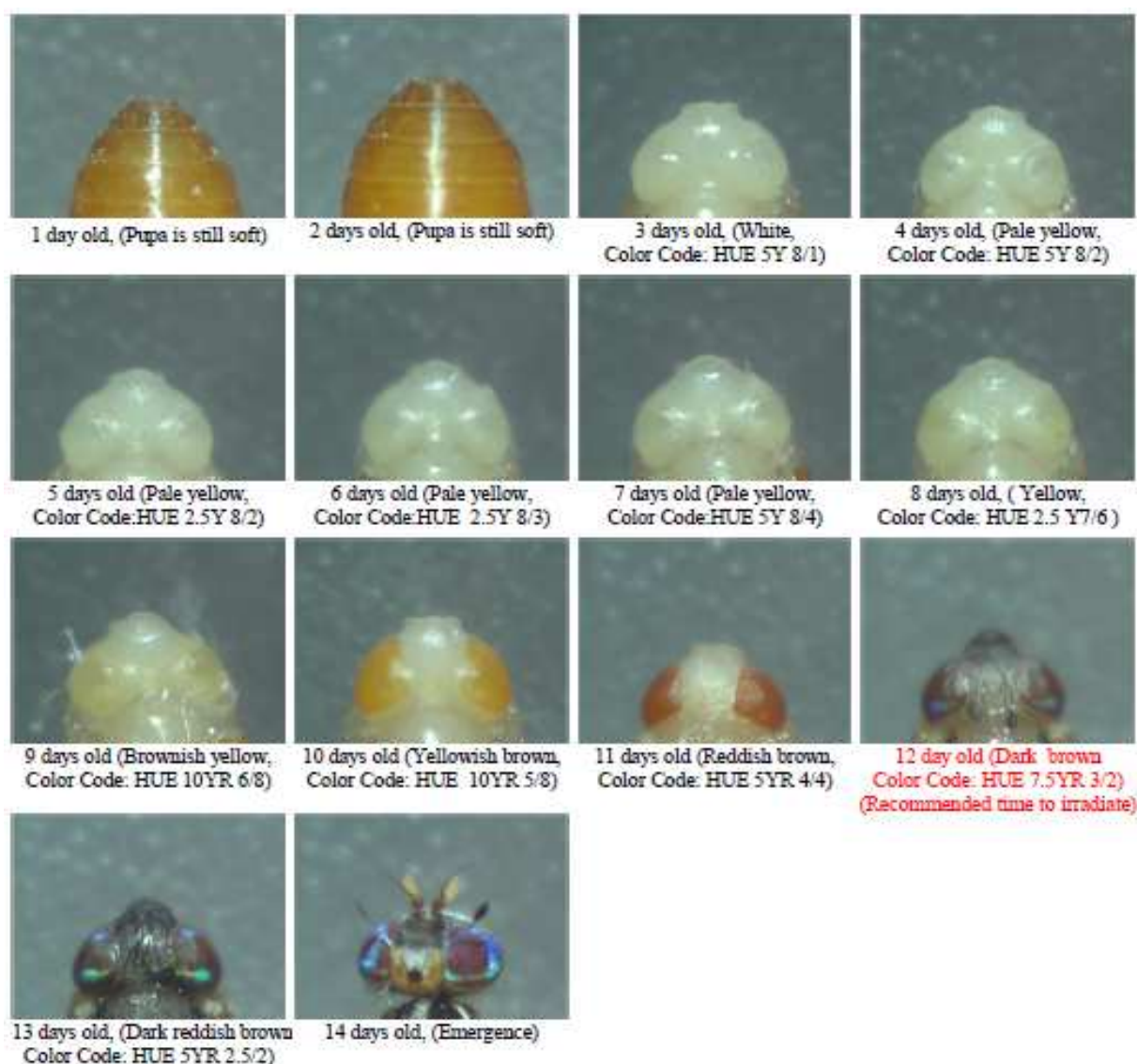


Figure 3.1. Eye colour change inside the puparium between pupation and adult emergence of *Ceratitidis capitata* flies (VIENNA-8 genetic sexing strain) at 20°C (for denomination of colour and code refer to the Munsell Soil Color Charts (Anonymous, 2000)).

3.2. Pre-irradiation Procedures

Marking of Insects

For most 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) 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
<i>Anastrepha fraterculus</i> (ARG)	57	25	22	14	13	21
<i>Anastrepha ludens</i>	49	-	33 ¹	16	15	16
<i>Anastrepha obliqua</i>	40	-	31 ¹	14	12	14
<i>Anastrepha serpentina</i>	49	-	31 ¹	16	14	15
<i>Bactrocera cucurbitae</i> (bisexual strain)	45	17	15	10	9	11
<i>Bactrocera cucurbitae</i> (wp-GSS MAR)	-	-	21 ¹	11 ²	9	10
<i>Bactrocera cucurbitae</i> (wp-GSS)	-	19	15	9	8	12
<i>Bactrocera dorsalis</i> (bisexual strain)	-	19	16	10	9	12
<i>Bactrocera dorsalis</i> (wp-GSS)	-	19	16	10	9	16
<i>Bactrocera invadens</i>	-	21	16	11	9	13
<i>Bactrocera oleae</i>	44	19	15	11	10	17
<i>Bactrocera tryoni</i>	-	-	18	11	9	13
<i>Bactrocera zonata</i>	-	-	21 ¹	11 ²	9	10
<i>Ceratitis capitata</i> (bisexual strain)	-	17	16	10	9	11
<i>Ceratitis capitata</i> (VIENNA-8)	-	17	14	9	8	15
<i>Ceratitis capitata</i> (VIENNA-8 SAF)	29	-	18 ¹	11 ²	9	-

¹19°C

²25°C

³Natural 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.

Plastic bottles. In the case of large pool-type irradiators (JS-7400), (for example at the *C. capitata* facility in Metapa, Mexico) pupae are held in 10- or 15-liter polypropylene bottles for irradiation.

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., N₂) 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 (%)
<i>Ceratitis capitata</i> reared at Tapachula, Mexico and shipped to emergence and release facilities (1980-81)			
Tapachuala	4	84.8	74.2
Guatemala	8	82.1	68.8
Los Gatos (CA, USA)	20	77.6	66.5
Flies reared and tested at the USDA Hawaii Fruit Fly Rearing Facility			
<i>Ceratitis capitata</i> (unirradiated)	0	95.6	92.5
	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			
<i>Anastrepha ludens</i>	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			
<i>Anastrepha obliqua</i>	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. Relevant Literature

Anonymous. 2000. Munsell® Soil Color Charts (Year 2000 Revised Washable Edition). Gretag Macbeth, New Windsor, New York, USA. 35 pp.

ASTM Standard E170. 2010. Standard Terminology Relating to Radiation Measurements and Dosimetry. ASTM International, West Conshohocken, PA. DOI:10.1520/E0170-10.

ASTM Standard E177. 2010 Standard Practice for Use of the Terms Precision and Bias in ASTM Test Methods. ASTM International, West Conshohocken, PA. DOI:10.1520/E0177-10.

ASTM Standard E456 2008. Standard Terminology Relating to Quality and Statistics. ASTM International, West Conshohocken, PA. DOI:10.1520/E0456-08E02.

ASTM Standard E1026 2004. Practice for Using the Fricke Reference-Standard Dosimetry System. ASTM International, West Conshohocken, PA. DOI:10.1520/E1026-04E01.

Bushland R.C. and D.E. Hopkins. 1951. Experiments with screw-worm flies sterilized by X-rays. J. Econ. Entomol. 44: 725-731.

Bushland R.C. and D.E. Hopkins 1953. Sterilization of screw-worm flies with X-rays and gamma rays. J. Econ. Entomol. 46:648-656.

Dodd B. and R.J. Vetter. 2009. Replacement of ¹³⁷Cs irradiators with X-ray irradiators. Health Physics. 96: 27-30.

FAO/IAEA. 2013a. Dosimetry for SIT: Standard Operating Procedure for Gafchromic® film dosimetry system for gamma radiation. V 2.0, 43pp.

- FAO/IAEA. 2013b.** Dosimetry for SIT: Standard Operating Procedure for Gafchromic® film dosimetry system for low energy X radiation. V 1.0, 48pp.
- Fisher, K. 1997.** Irradiation effects in air and in nitrogen on Mediterranean fruit fly (Diptera: Tephritidae) pupae in Western Australia. *J. Econ. Entomol.* 90(6): 1609-1614.
- IAEA. 1992.** Laboratory training manual on the use of nuclear techniques in insect research and control. Third edition. Technical Reports Series No. 336. IAEA, Vienna. Austria. 183pp.
- ISO/ASTM Standard 51275:2013.** Practice for Use of a Radiochromic Film Dosimetry System. ASTM International, West Conshohocken, PA. http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=62954.
- ISO/ASTM Standard 51539:2013.** Guide for Use of Radiation-Sensitive Indicators. ASTM International, West Conshohocken, PA. http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=63642.
- ISO/ASTM Standard 51940:2013** Guide for Dosimetry for Sterile Insect Release Programs. ASTM International, West Conshohocken, PA. http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=61087.
- Kuehni, RG. 2002.** "The early development of the Munsell system". *Color Research and Application* 27 (1): 20–27. doi:10.1002/col.10002.
- Mastangelo T, A.G. Parker, A. Jessup, R. Pereira, D. Orozco-Davila, A. Islam, T. Dammalage and J.M.M. Walder. 2010.** A new generation of X ray irradiators for insect sterilization. *J. Econ. Entomol.* 103(1): 85-94.
- Munsell, A.H. 1905.** A Color Notation. Boston: G. H. Ellis Co
- Munsell, A.H. 1912.** "A Pigment Color System and Notation". *The American Journal of Psychology* 23 (2): 236–244. doi:10.2307/1412843.
- Nestel D., E. Nemny-Lavy, S. M. Islam, V. Wornoyaporn and C. Cáceres. 2007.** Effects of pre-irradiation conditioning of medfly pupae (Diptera: Tephritidae): Hypoxia and quality of sterile males. *Fla. Entomol.* 90: 80-87.
- Nickerson, D. 1976.** "History of the Munsell color system, company, and foundation". *Color Research and Application* 1 (1): 7–10.
- Parker A.G. and K. Mehta. 2007.** Sterile insect technique: a model for dose optimization for improved sterile insect quality. *Fla. Entomol.* 90: 88-95.
- Ruhm, M.E. and C.O. Calkins. 1981.** Eye-color changes in the Mediterranean fruit fly *Ceratitis capitata* pupae, a technique to determine pupal development. *Entomol. Exp. Applicata.* 29: 237-240.
- Sotero R. and R. Pereira. (in press).** Age and temperature related pupal eye colour changes in various tephritid fruit fly species with a view to optimizing irradiation timing. *International Journal of Tropical Insect Science.*
- Zavala, J.L., M.M. Fierro, A.J. Schwarz, D.H. Orozco and M. Guerra. 1985.** Dosimetry practice for the irradiation of the Mediterranean fruit fly *Ceratitis capitata* (Wied.). 23-30. *In* IAEA [ed.] High dose dosimetry, Proceedings of the International Symposium, IAEA STI/PUB/671, IAEA. Vienna, Austria.

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.

Selection of a suitable dosimetry system depends on several considerations, including dose range of interest, ease of measurement, expertise available, environmental factors that can be important at the location of use, cost and uncertainty that is consistent with the process. Considering these factors, a radiochromic film dosimetry system seems to be the most suitable for SIT programmes. These film dosimeters (generally 20-100 μm thick) change colour on irradiation. The colour change, which depends upon the dose, is measured by a spectrophotometer at a selected wavelength or a simpler fixed wavelength photometer. There are several such systems, and the Gafchromic® dosimetry system is selected based on the specific requirements of SIT programmes. This system offers SIT practitioners and their clients a relatively simple, low cost and accurate means of assessing dose. The dosimeter is a small (about 1x1 or 2x2 cm square), thin (± 100 micrometer) film which changes colour under irradiation. Relevant dosimetry procedures as well as various components of this dosimetry system are described in the documents "Dosimetry for SIT: Standard Operating Procedure for Gafchromic® film dosimetry system for gamma radiation, v 2.0", and "Dosimetry for SIT: Standard Operating Procedure for Gafchromic® film dosimetry system for low energy X radiation, v 1.0" (FAO/IAEA 2013a, b)¹. The quality of dosimetry and hence the success of the sterilisation process depends on rigorously following these SOPs. They also include examples of all the data forms necessary for dosimetry.

There are also accompanying spreadsheets in Microsoft Excel format containing all necessary forms, with formulae to do the pertinent calculations automatically (the files contains no macros). Instructions are included in a separate document for the use of the spreadsheets. The spread sheets and the instructions are available from the Insect Pest Control Section

¹ These document are referred to several times in this chapter. For convenience, they are referred to as 'the SOP(s)' henceforth. The appropriate SOP should be consulted depending on whether a gamma or low energy X ray irradiator is being used.

(<http://www-naweb.iaea.org/naifa/ipc/public/ipc-gafchromic-dosimetry-sterile-insect-technique.html>).

Even though this dosimetry system can be used for various types of radiation, including electrons, the procedures described here are limited to gamma radiation emitted by either ^{60}Co or ^{137}Cs and low energy X rays.

The remaining sections describe some of the salient points for the following procedures for establishing and using the Gafchromic® dosimetry system for measurement of dose:

- Procedure for setting up and using Gafchromic® dosimetry system;
- Procedure for establishing traceability to a national/international standard;
- Procedure for establishing the calibration relationship for the dosimetry system;
- Procedure for determination of uncertainty in the measured dose value using the dosimetry system.

4.1. Dosimetry System

The Gafchromic® dosimetry system consists of:

- Gafchromic® film dosimeters.
- DoseReader2® plus accessories or Radiachromic® reader plus accessories (see **Figure 4.1**).
- Procedure for its use.

The procedure for using the dosimetry system and the characteristics of the components are described here. More general information on radiochromic film dosimetry systems is available from references listed under Relevant Literature.

Gafchromic® Film Dosimeters (HD-V2)²

a) *Description:*

The film is about 0.1 mm thick, and is transparent and pale yellow before irradiation. The active layer of the film contains two dyes, a non-reactive yellow dye and a reactive blue dye. The reactive dye turns darker blue upon exposure to ionising radiation. However, the optical density (OD) of the film increases (the blue colour deepens) slightly with time after exposure so the irradiated film is read about 20 to 28 hours after irradiation. The OD of the irradiated dosimeter film (for the same dose) also depends to some extent on the temperature of the dosimeter during irradiation as well as during reading. The non-reactive yellow dye is used to correct for variation in the active layer thickness. The DoseReader2 incorporates two light sources to read the film simultaneously in the blue and amber parts of the spectrum. The final value used to estimate the dose is the ratio of the reading in amber to the reading in blue (referred to below as OD). The film may also be read using a Radiachromic® Reader but as this can only read one colour at a time (600 or 510 nm) it is not possible to correct for variations in the active layer thickness.

² Supplied by International Specialty Products (ISP), New Jersey, U.S.A. Gafchromic® is a registered trade mark.

b) *Handling:*

For reliable analysis, it is essential that the central portion of the film through which the analysing light passes is kept clean. The film should be held by the edge with a pair of tweezers (preferably with fine points) so as not to leave any fingerprints on the film. It is preferable to wear thin polythene or latex (surgical) gloves for this activity. The film dosimeter is purchased as a large sheet (c. 20 cm x 25 cm), and is cut into small dosimeters of about 10 mm x 10 mm, since the reader can accept films of this size only. For X ray a 20 x 20 mm dosimeter is used that must be cut before reading into quarters to fit into the reader. After cutting, use a small paper envelope to store each film dosimeter and remove it only for OD measurement. When measuring x-ray dose, the dosimeters must be irradiated in an envelope to provide a consistent material in close proximity to the dosimeter as the dosimeter is very sensitive to its immediate environment for low energy photons. All dosimeters should be stored in an envelope to keep them free of any dust or other contamination. Light, especially UV, can induce colour change in the film simulating radiation dose so the films should not be exposed to sunlight and exposure to room lights (especially fluorescent lights) should be minimized. The remaining sheet should be stored in its envelope when not in use away from the irradiator so that it is not exposed to radiation.

c) *Background-OD:*

Since only the radiation-induced colour change is related to dose the OD of the un-irradiated film, also called 'background-OD', must be measured. This value must be determined for each lot of dosimeters following this procedure. Prepare ten dosimeters (10 mm x 10 mm) from the dosimeter sheet. Measure the OD of each dosimeter and calculate the mean value; this is background-OD. This is applied to all dosimeters of that lot. If the lot lasts longer than 6 months, the background-OD may change and this procedure should be repeated to update the value of the background-OD.

Radiachromic® Reader³

a) *Description:*

The Radiachromic® reader, FWT-92 (**Figure 4.1**) is a photometric instrument for the read-out of radiochromic dosimetry films, such as Gafchromic®. It displays digitally the OD value of the film being read. It has an option for selecting the wavelength of the analysing light, either 510 or 600 nm; the latter should be used for reading the Gafchromic® HD-810 films.

Note: Operators are encouraged to read carefully the entire Operations Manual of the reader provided by the manufacturer before using the reader. As the Manual was developed for a different film, FWT-60, there may be minor differences which are noted in the SOPs.

³ Supplied by Far West Technology, Inc., California, U.S.A. Radiachromic® is a registered trademark.



Figure 4.1: Radiachromic® reader.

b) *Set-up:*

For optimum operation, the reader should be placed in the laboratory at a location that is dust-free, away from direct sunlight and any air-draft, and where the temperature is relatively constant throughout the year. The absorbance scale of the reader should be checked periodically (daily when the reader is in use) against neutral density filters.

c) *Adjustment of the absorption scale (HI/LO check):*

It is essential for the correct functioning of the reader that the absorption scale is adjusted regularly. For the high end of the scale, lift the dosimeter holder slightly (1-2 mm) from its well, rotate it through about 45°, and then release it. The 'HI' knob should then be adjusted to *just* display 9999 (and not 0.999), approaching from low numbers like 3.01. This sets infinite OD or 0% transmission. For the low end of the scale, lift the holder again from its well slightly and rotate it back to its normal position and then release it. Now the 'LO' knob should be adjusted *just* to read 0.000, approaching from non-zero numbers. This sets 0 (zero) OD or 100% transmission. (Note: If the low value cannot be set, the lamp may be too dim or burnt out and needs replacement.) Repeat the above steps until both readings are correct, that is, no further adjustments are needed.

d) *Maintenance of the absorption scale:*

Use the set of three neutral density (ND) filters⁴ to check the consistent operation of the reader (absorption scale). Each filter should be placed in the well of the reader replacing the dosimeter holder, and its OD value measured. The nominal values are approximately: 0.3 (for the filter marked yellow), 0.6 (green) and 1.1 (blue). The actual values will vary slightly from

⁴ Supplied by Far West Technology, Inc., California, U.S.A.

reader to reader. Initially, these values should be measured during the calibration of the dosimetry system. Later, these values should be checked at least once a day when the reader is in use. Best time is before starting measurements of the dosimeter films. Note: The OD values should be the same (the last digit may change by 1 or 2) as that during calibration of the dosimetry system. Different values indicate a potential problem with the reader. Refer to the Operations Manual of the reader for troubleshooting.

Procedure

a) *Routine procedure for optical density (OD) measurement:*

The procedure listed below should be followed routinely to measure optical density (OD) of a film dosimeter:

- Check that the reader is set up as specified.
- Check that the selected wavelength is 600 nm.
- Before any OD measurements, perform the HI/LO check and repeat it every 30 minutes during the measurement session.
- Measure the OD of the three ND filters at least once a day, preferably before starting the measurements with the dosimeter films. Compare these OD values with those measured during the calibration of the dosimetry system. Different values indicate a problem with the reader.
- Insert the dosimeter into the holder and place the holder in the reader. The interior of the reader will be above room temperature due to the heat generated by the light bulb, which may cause the OD reading to increase. Observe the OD reading until it has been stable for 10 seconds.
- Record the OD of the dosimeter.
- Always replace the dosimeter holder in its well in the reader when not in use to prevent entry of dust. This will also maintain the holder at the internal temperature of the reader, reducing the time for new readings to stabilize.

DoseReader2®⁵

e) *Description:*

The DoseReader2® is a photometric instrument for the read-out of radiochromic dosimetry films, such as Gafchromic®. It displays digitally the OD value of the film being read at two wavelengths, amber (590 nm) and blue (465 nm) and automatically transfers the readings via a USB cable to a computer with the appropriate software installed each time a dosimeter is present in the slot and the lid is closed. The simultaneous reading at two wavelengths permits a correction to be made for variation in the thickness of the active layer on the Gafchromic® film. The data is transmitted in ASCII format with a tab character between fields and a CR/LF pair at the end of each data set. When the focus of the computer is in an Excel spread sheet the data will be laid out in the spread sheet in three columns. When initially booted up, the DoseReader2 transmits a header with the serial number of the reader and column headings. For each film read it transmits the OD at the blue wavelength, the OD at the amber wavelength and the temperature of the read head to allow for compensation for the

⁵ Supplied by RadGen, Hungary need full details

temperature dependence of the film. The spread sheet should be used to calculate the ratio of amber to blue for each reading to give the final OD value that will be inserted into the dosimetry spread sheet.

The reader is powered from the USB port of the computer or from an independent USB power supply if a computer is not used to record the values.

Note: Operators are encouraged to read carefully the entire Operations Manual of the reader provided by the manufacturer before using the reader.

f) *Set-up:*

For optimum operation, the reader should be placed in the laboratory at a location that is dust-free, away from direct sunlight and any air-draft, and where the temperature is relatively constant throughout the year. The absorbance scale of the reader should be checked periodically (daily when the reader is in use) against neutral density filters.

If the automatic data transfer to a computer is to be used, the software should be installed on a suitable computer following the manufacturer's instructions. In this manual it is assumed that a computer is being used. If the data does not appear on the computer, follow the operations manual troubleshooting guide.

g) *Maintenance of the absorption scale:*

Use the supplied set of three or four neutral density (ND) filters to check the consistent operation of the reader (absorption scale). Each filter should be placed in the slot of the reader and its OD value measured. The nominal values are approximately: 0.5, 1.0, 2.0 and 3.0 (if supplied). For the measurement of the ND filters, either the reading in amber or the reading in blue is used, not the ratio of amber to blue. The actual values will vary slightly from reader to reader and depending on which colour is used. Initially, these values should be measured during the calibration of the dosimetry system. Later, these values should be checked at least once a day when the reader is in use. Best time is before starting measurements of the dosimeter films. *Note:* The OD values should be the same (the last digit may change by 1 or 2) as that during calibration of the dosimetry system. Different values indicate a potential problem with the reader. Refer to the Operations Manual of the reader for troubleshooting.

Procedure

b) *Routine procedure for optical density (OD) measurement:*

The procedure listed below should be followed routinely to measure optical density (OD) of a film dosimeter:

- Check that the computer is correctly configured and that the data capture software is running.
- Open a blank spread sheet and position the cursor in the second cell of the first row (cell B1). Make sure the focus remains in the spread sheet; if the focus is changed to another window the data will be lost.
- Ensure that there is no dosimeter in the reader and that the reader lid is closed. Plug the reader into the USB port of the computer used during the initial setup and wait for it to boot up. The header information should appear in the spread sheet; if it does not follow the troubleshooting instructions.

- The first column of the spread sheet may be used to record information about the dosimeter to be read. After entering this information make sure the cursor is in the second column one cell below the heading or last reading.
- Wait a few minutes for the reader to stabilize then press either button to initiate the recording of the readings without any dosimeter (blank reading). The values appears on the display of the DoseReader® but are not transferred to the computer.
- To measure a film open the lid, place the film in the slot and close the lid. The reader will immediately start to measure the reading at the two colours, amber and blue, and an indicator at the bottom right of the display will count down and back up during the reading. Once this is complete the reader beeps and the data is automatically transferred to the computer. Open the lid and remove the dosimeter.
- If a computer is not being used, record the two OD values of the dosimeter.
- About each ten minutes, close the lid with no dosimeter present and wait for a new blank reading. The reader beeps when the blank reading is complete. No data is transferred to the computer
- Measure the OD of the three ND filters at least once a day, preferably before starting the measurements with the dosimeter films. Compare these OD values with those measured during the calibration of the dosimetry system. Different values indicate a problem with the reader.
- Always close the lid when the reader is not in use to prevent entry of dust. This also allows the DoseReader® to update the blank reading.

4.2. Establishing Traceability

General

A system of calibration should exist within each country to ensure that all dose measurements can be related to the national standard, and through that to the international measurement system, through an unbroken chain of comparisons. Such a chain is known as the *traceability chain*. Without such traceability, the measurement does not have much validity. The most common method of establishing traceability is to determine the dose rate value at a reference point in the radiation field. For gamma irradiation this is achieved by means of transfer-standard dosimeters supplied and analysed by a standards laboratory and for X ray by use of an ion chamber and electrometer calibrated by a standards laboratory (calibration of alanine transfer dosimeters for low energy X ray is in progress and a transfer dosimetry system may become available shortly that would avoid the need for ion chambers). It is important that the irradiation geometry and irradiation conditions at this reference point are reproducible.

Transfer-standard dosimeters (for gamma calibration)

The transfer-standard dosimeters must be from a recognised or accredited laboratory or a national laboratory. Several laboratories provide this service, for example, NIST in USA, NPL in UK, RISØ in Denmark. The International Atomic Energy Agency no longer offers dose assurance services.

Transfer-standard dosimeters may be obtained from an accredited National Standards Laboratory or from one of the following:

National Institute of Standards and Technology

100 Bureau Drive, Stop 8460
Gaithersburg, MD 20899-8460

Fax: 301/869-7682

Technical Contacts:

James M. Puhl

Tel: 301/975-5581

E-mail: james.puhl@nist.gov

<http://ts.nist.gov/MeasurementServices/Calibrations/dosimetry.cfm>

Note: Please contact the technical staff before shipping instruments or standards to the address listed above.

National Physical Laboratory

(<http://www.npl.co.uk/ionising-radiation/dosimetry/products-and-services/mail-order-alanine-reference-dosimetry-service-for-radiation-processing/6027>)

Customer Service tel: +44 20 8943 8695

E-mail: dosimetry_enquiries@npl.co.uk

Risø National Laboratory for Sustainable Energy

Technical University of Denmark

Frederiksborgvej 399

P.O. Box 49

DK-4000 Roskilde

Tel. (Risø main number) +45 4677 4677

Fax +45 4677 5688

http://www.risoe.dtu.dk/Risoe_dk/Home/business_relations/Products_Services/Dosimetri/NUK_highdose_measurment_service/alanine_pellets.aspx

All three laboratories are national institutes of standards recognized by the International Organization for Standards (ISO). A fee is assessed to cover the cost of these calibration services.

The preferred transfer-standard dosimeter to be used for gamma irradiators is alanine. After receiving the transfer-standard dosimeter set from one of the above laboratories, it should be irradiated at a reference point in the radiation field following the procedure given below in combination with the instructions provided by the laboratory from which the dosimeters are obtained. After irradiation, the dosimeter set should be returned to the appropriate laboratory for analysis along with the data sheets. The laboratory will then analyse the dosimeters and issue a certificate stating the dose received by the dosimeters and the uncertainty in that value.

Calibrated ion chamber and electrometer (for X ray calibration)

Transfer-standard dosimeters are currently not available for the low photon energy of low-voltage X irradiators (40-150 keV). For these systems the dose rate is measured with an ion chamber and electrometer. In an ion chamber a high voltage is placed across a chamber of defined volume. When an energetic photon passes through the chamber it generates free electrons that are attracted to the anode causing a current to flow through the electrometer to the cathode. The electrometer measures the current, which is directly proportional to the

photon field. For most X ray systems a Farmer type chamber is most appropriate (a cylindrical chamber that responds equally to photons from any direction about the mid plane of the chamber). The chamber volume should be quite small, about 0.1 - 0.6 cm³, so that the calibration reading is obtained from a small volume where the dose rate will vary little. It is also recommended to use a free air chamber with pressure and temperature calibration. The ion chamber, electrometer and any associated electronics and components should be calibrated to a recognized standard. The calibration will show the sensitivity and the uncertainty in the reading at different photon energies.

Reference Irradiation Conditions

The placement of the transfer dosimeter set or ion chamber at a fixed reference point should be consistent to achieve reproducible results. This can be conveniently achieved by using a specially designed build-up holder, a support stand and arranging a set of reference marks in the irradiation chamber so that the holder can be placed in exactly the same position each time. The build-up holder has the added function of producing a standard electron cascade (build-up) independent of any other material in the chamber so that the dosimeters are exposed to consistent conditions.

A self-contained irradiator, such as Nordion or Sheppard Gammacell or Husman irradiator, consists of several radioactive pencils generally arranged in a cylindrical array. The sample or a dosimeter to be irradiated is located (stationary or rotated) in the middle of the cylindrical irradiation chamber where the dose rate is high and relatively uniform. For this type of irradiator, the most suitable build-up holder is an open cylinder (like a cup). The SOP describes an example of such a build-up holder which has the inside diameter of about 27 mm (just large enough to accommodate three dosimeters, each being about 12 mm in diameter). It should be made of any polymeric material not containing chlorine, such as PMMA. The build-up holder should be located in the irradiation chamber so that the dosimeters are at the centre of the radiation field, where the dose rate is most uniform. Also, some method should be available to position this holder always at the same location. The SOP also describes the design of a support stand suitable for Nordion Gammacell 220 for this purpose.

A panoramic irradiator consists of several radioactive pencils arranged in a plane or as a single rod. For routine operation, the sample is either located stationary in front of the source and rotated, or it passes by or around the source. Even though the sample (for example, pupae canister) may be moving past the radiation source for routine irradiation, it may be necessary in some cases that the dosimeters are *stationary* for the dose-rate measurement and also for calibration irradiations (see 4.3. Calibration of Gafchromic® Dosimetry System). For such irradiators, the build-up holder is flat and is placed in such a way that the flat surface is perpendicular to the radiation beam. An example of such a build-up holder is given in the SOP. It should be located at a reference point that is at approximately the same distance from the source as the sample is during routine irradiation. Also, some method should be available to position this holder repeatedly at the same location in the radiation field.

For an X irradiator, the ion chamber must be fixed in the centre of one of the treatment canisters and the canister filled with pupae or a suitable load material – “instant” rice has been found to make a suitable dummy for fruit fly pupae, having a similar chemical composition and bulk density. For this a suitable holder must be constructed that allows the ion chamber to

be fixed at a known location and subsequently allows the dosimetry system to be exposed at the same location. Details of a suitable holder for the RS2400 are in the SOP. As the ion chamber must be connected to the electrometer via a cable sample rotation must be switched off. If a free air ion chamber is being used the chamber should be protected in a rubber sleeve, such as the finger cut from a latex glove, to prevent the air hole becoming blocked but connection with the external atmosphere must be maintained to ensure the correct pressure in the ion chamber.

Irradiation

Alanine dosimeters are available in several formats, the commonest of which is a small, flat pellet, about 3mm in diameter. The pellets need to be enclosed in a suitable holder to match the film holder described in the SOP for holding the Gafchromic film, 12 mm in diameter and 55 mm high. This should be requested from the transfer standard dosimeter supplier so as to avoid the need to handle the alanine pellets directly. Three such holders, each usually containing three pellets, constitute a set. For a cylindrical build-up holder, all three dosimeter holders may be positioned in a triangular fashion within the build-up holder. For a flat build-up holder, the three dosimeter holders are placed in the three holes of the build-up holder. The build-up holder should then be carefully placed at the reference point in the radiation field. The selected dose value is a compromise between several requirements:

- Alanine dosimetry requires that the dose should be greater than 600 Gy.
- The dose should be large enough so that the transit dose⁶ is negligible (i.e. < 0.5%). (see “Transit Dose” in 4.3. Calibration of Gafchromic® Dosimetry System.
- The dose should be as small as possible so that the temperature of the dosimeter does not change significantly during irradiation.

Optimum value for irradiation is discussed in the SOP for several irradiators. The irradiation time is then estimated from an approximate value of dose rate at the reference point. Ensure that the timer used for time measurement is calibrated with traceability. See manufacturer’s documentation on how to check timer accuracy. The temperature of the dosimeter should be controlled or monitored during irradiation. If possible, control the dosimeter temperature during irradiation at 25°C; this is the temperature at which alanine dosimeters are normally calibrated. If control of the temperature is not possible, the dosimeter temperature must be measured or estimated *just* before irradiation (minimum temperature) and *immediately* after irradiation (maximum temperature) by temporarily introducing a standard thermometer inside the build-up holder next to the dosimeters.

Dose Rate at the Reference Point

The Standard Dosimetry Laboratory will analyse the dosimeters and return the results as a certificate. It contains information regarding dose received by the dosimeters and the uncertainty in this value. Using this value of the dose and the time of irradiation, dose rate at the irradiation position can be calculated. Here it is assumed that the transit dose is insignificant compared to the delivered dose. Dose rate is generally expressed in units of Gy/s, Gy/min or kGy/hour. Note that this value is valid for the specific irradiation conditions

⁶ Transit dose is the dose received by the dosimeter when the source or the dosimeter is moving.

at the reference point and for the day of irradiation. However, it is independent of the irradiation temperature.

Frequency

The dose rate should be measured every 3 to 4 years by using transfer-standard dosimeters from a standards laboratory. It may be necessary to do it earlier if any relevant part of the irradiation system is altered, such as replenishment of the source, or modification to movement mechanism or irradiation set up that can affect the dose rate.

4.3. Calibration of Gafchromic® Dosimetry System

Calibration is the relationship between the absorbed dose and the resulting response of the dosimeter (in this case, it is the change in optical density, OD) for specific irradiation conditions (such as dosimeter temperature during irradiation). A dosimetry system must be calibrated before it can be used to measure dose.

Calibration procedure for a dosimetry system consists of:

- Irradiating several dosimeters at the reference point (where dose rate is accurately known) at specified dose levels.
- Measuring the OD of the irradiated dosimeters with the reader and determining the response for each dosimeter.
- Establishing a relationship between response and dose.

These steps are discussed thereafter.

Irradiation

It is essential that the Gafchromic® dosimeters are irradiated at the same reference point where the dose rate was determined with the transfer dosimeters or ion chamber, and with the same irradiation geometry and conditions. Thus, the *same* build-up holder should be used for gamma calibration. Since films are physically different than alanine dosimeters, a special film holder is necessary. An example of such a dosimeter holder is given in the SOP. It consists of a solid rod of PMMA of 12-mm diameter and 50-mm long. A long cut is made along the length for inserting a dosimeter film-strip 9 mm x 50 mm. Three such film holders (with the Gafchromic® films) are then placed in the build-up holder (cylindrical or flat depending on the type of irradiator). The dosimeter holder then should be placed exactly at the same location as it was during transfer standard dosimeter irradiation. For X ray a suitable holder must be used to place the film at the centre of the position occupied by the ion chamber.

Several dose values should be selected within the useful dose range; exact values depend on the type of insect. For fruit flies, calibration irradiation should be performed at 6 dose levels nominally set at: 20, 60, 100, 140, 180 and 220 Gy. The corresponding irradiation times can be calculated from the known dose rate (from the transfer dosimeter report or ion chamber) on

the day of irradiation⁷. The dosimeters are then irradiated for these times or some convenient times close to these values may be selected. As mentioned earlier, it is essential to control the dosimeter temperature during irradiation, or to monitor it continuously, or to measure the minimum temperature (*just* before starting irradiation) and the maximum temperature (*immediately* after irradiation). If possible, all irradiations should be carried out at approximately the same temperature.

Calculate the effective value of the dosimeter temperature, T_{eff} by averaging the measured values for each irradiation. If all the irradiations were not performed at the same temperature, calculate the calibration temperature, T_{cal} as the mean of all the T_{eff} values determined above. This T_{cal} value is later used for correcting the measured OD values.

Response Determination

After irradiation, it is necessary to cut the irradiated film-strip or square into three or four dosimeters of size 10 mm x 10 mm, so as to fit them in the reader. Wait for about 20 to 28 hours before measuring the OD of these dosimeters using the procedure given in “Routine Procedure for Optical Density (OD) Measurement” in 4.1 Dosimetry System. There are several dosimeters which were irradiated at the same dose. Calculate the mean and standard deviation for the OD values for these dosimeters for each dose. The coefficient of variation (CV) at each dose may be calculated by:

$$CV(\%) = (\text{standard deviation} / \text{mean}) \times 100$$

The value of CV should not be more than 2% for each dose.

The measured OD value needs to be corrected for the background-OD and for dosimeter temperature during irradiation. Thus, response is calculated as:

$$\text{Response} = (\text{Measured OD} - \text{Background OD}) (1 - 0.0073 \times \Delta T),$$

where, $0.0073 \text{ } ^\circ\text{C}^{-1}$ = temperature coefficient for Gafchromic® dosimeter (ISP 1999), and:

$$\Delta T (^\circ\text{C}) = T_{\text{eff}} - T_{\text{cal}}.$$

Transit Dose

Transit dose is defined as the dose a sample or dosimeter receives while either the source or the sample/dosimeter is moving. It is essential to estimate the value of transit dose for the irradiator being used for the SIT programme. There are two reasons for this: (1) the transfer dosimeter dose should be such that the transit dose is insignificant in comparison, and (2) for calibration of Gafchromic® dosimetry system, the correct value of dose received by the dosimeters must be known. For X ray, the equivalent of the transit dose comes from the build-up of the voltage and current in the first few seconds after the system is energized. If calibration is made against kW it is not necessary to calculate transit dose; see the SOP for details.

⁷ The dose-rate value on the day of irradiation should be calculated based on the dose-rate value measured with the transfer-standard dosimeters and the time interval since then. This calculation requires the half-life value of the radioactive source; this value for cobalt-60 is 5.271 years (Unterweger *et al.* 1992) and for Cesium-137 is 30.07 years (Tuli 1994). This calculation is not required for X ray.

If the transit dose is less than 0.5% of the minimum irradiation dose (for example, for fruit flies the minimum dose for calibration is 20 Gy; and 0.5% is 0.1 Gy), then the transit dose may be completely ignored. Considering this, transit dose may be ignored for Gammacells with dose rate less than about 2 Gy/min. For irradiators with higher dose rate, a detailed procedure is given in the SOP for the estimation of transit dose.

This value of transit dose should be added to each of the 6 nominal dose values (namely, 20, 60, ..., ..., 220 Gy) to determine the 'actual dose' value received by the dosimeters. Thus:

$$\text{Actual dose} = (\text{dose rate on the day of irradiation}) \times (\text{irradiation time}) + \text{transit dose}.$$

Calibration Relationship

The objective here is to determine the relationship (generally, mathematical) between the dosimeter response (y-axis) and the actual value of dose received by the dosimeter (x-axis). This is achieved most conveniently by regression analyses; the SOP uses this procedure and the required spreadsheet is available from the Insect Pest Control Section (IPCS) (FAO/IAEA 2008).

For the case of fruit flies, this relationship is almost linear, but quadratic fit may be better. The calibration relationships may be described as:

Linear function: $\text{Response} = a + b (\text{Dose})$

Quadratic function: $\text{Response} = c + d (\text{Dose}) + e (\text{Dose})^2$

The selection between the two can be made by observing the distribution of the percentage residuals⁸ for the two cases following the procedure given in the SOP. This calibration relationship is valid for the specific lot of dosimeters for one year, and for the temperature employed for the irradiations, T_{cal} . The spread sheet provided by the IPCS provides additional fit curves; the one giving the lowest residual value should be selected.

Frequency

The dosimetry system should be calibrated every year, or sooner if any part of the dosimetry system is changed, such as new lot of dosimeters or repairs to the reader. [Note: a lamp change in the Radiachronic® reader does not require re-calibration]

Use of the Calibration Relationship

To measure the dose at a point, follow the procedure given below.

Irradiation:

1. Place one or more 10 mm x 10 mm dosimeter (3 dosimeters are preferable) for gamma or a 20 x 20 mm dosimeter for X ray from the calibrated lot at each point of interest. Use a small envelope for the dosimeter(s) and write the relevant identification and information on the envelope. More than one dosimeter may be placed in one envelope for gamma but only one dosimeter may be placed in an envelope for X ray.
2. Irradiate the sample (with the dosimeters in the envelope).

⁸ For each response value, residual is calculated as follows: $\text{Residual}(\%) = 100 \times (D_{\text{cal}} - D_{\text{actual}}) / D_{\text{actual}}$, where D_{actual} is the actual value of dose and D_{cal} is the calculated value of dose for the corresponding response value using the calibration relationship.

3. Estimate the effective temperature (T_{eff}) of the dosimeter during irradiation (see “Uncertainty in the dose value arising from uncertainty in the irradiation temperature during dose measurement, u_{temp} ,” in 4.4 Uncertainty).

OD measurement: [Note: These measurements are made about 20-28 hours after irradiation]

4. Follow the procedure in “Routine Procedure for Optical Density (OD) Measurement” in 4.1 Dosimetry System to measure the OD of the dosimeter(s).
5. Determine response for each dosimeter following the expression given in “Response Determination” (this section).

Dose determination:

6. Determine dose using the value of the response and the calibration relationship determined in “Routine Procedure for Optical Density (OD) Measurement” in 4.1 Dosimetry System.

4.4. Uncertainty

Uncertainty reflects the degree of variability in the measured value, and thus it is essential to evaluate it. First, the sources of uncertainty should be identified, and their effects minimized as much as possible; and then the remaining ones should be evaluated. This is most easily done by dividing and sub-dividing the procedures for calibration and use of the dosimetry system into smallest activities, and then assessing what uncertainties are likely to be associated with each of them. The uncertainty associated with the dose measurement can then be calculated by combining the individual components together.

For Gafchromic® dosimetry system, the total uncertainty in the measured dose value consists of the following components [Note: all these components are in %].

Uncertainty in the Dose Value due to Uncertainty in the Dose-rate Value, u_{dr}

Uncertainty in the dose-rate value at the reference point is transferred to the uncertainty in the measured dose value. This value is available on the certificate from the national laboratory that provided the transfer-standard dosimeters or from the ion chamber calibration certificate (see “Dose Rate at the Reference Point” in 4.2 Establishing Traceability).

Uncertainty in the Dose Value Arising from the Calibration Relationship, u_{fit}

This represents the uncertainty arising from the fitting procedure and is based on the values of the residuals (see “Calibration Relationship” in this section). Detailed procedure for calculating this value is described in the SOP. In brief,

$$u_{\text{fit}} = \{\Sigma(\text{Residual}(\%))^2 / n\}^{1/2},$$

where; n is the total number of data points in the calibration relationship, and the summation to be carried over all these n values.

Uncertainty in the Dose Value Arising from Non-homogeneity in the Dosimeter Lot, u_{lot}

Response to dose of all dosimeters in a lot is not exactly the same. This non-homogeneity adds to the uncertainty in the measured dose. This value is determined by irradiating several

(say 10) dosimeters selected randomly from the lot. They should be irradiated to the same dose under identical conditions and then analysed in a similar way. They should be irradiated *together* (if possible, but in separate envelopes for x ray) at about 100 Gy. Calculate the mean and standard deviation of the response values, and determine coefficient of variation CV(%). The uncertainty component $u_{lot} = CV(\%)$. However, if n dosimeters are used at one location to measure dose, the uncertainty in the mean value is reduced by \sqrt{n} . Thus, u_{lot} for n dosimeters $= CV(\%)/\sqrt{n}$.

Uncertainty in the Dose Value Arising from Uncertainty in the Irradiation Temperature during Dose Measurement, u_{temp-i}

As discussed in “Response Determination” (this section), the measured OD value is corrected for irradiation temperature if it differs from that during calibration. However, if the irradiation temperature is not known accurately, this procedure introduces uncertainty in the correction applied. Assuming that the effective dosimeter temperature, T_{eff} during irradiation is known to be between T_{min} and T_{max} , $u_{temp-i} = 0.73 \{(T_{max} - T_{min})/2\}/\sqrt{6}$. The factor of $\sqrt{6}$ is based on the assumption that the effective temperature has triangular probability distribution within the two limits (ISO 1993). Note: if, for example, $(T_{max} - T_{min}) = 10^\circ\text{C}$, $u_{temp-i} = 1.5\%$.

Uncertainty in the Dose Value Arising from Uncertainty in the Dosimeter Temperature during OD Read-out, u_{temp-r}

The measured OD value depends on the dosimeter temperature during read-out. If this value differs from the value during calibration of the dosimetry system, a correction needs to be applied to the measured OD value. The dosimeter temperature during read-out depends, to some extent, on the ambient temperature surrounding the reader, and hence the need to ensure that the laboratory temperature is as uniform as possible throughout the year. The measured OD can be corrected if the dosimeter temperature during read-out is known (supplied by the DoseReader2®), however it is difficult to measure this in a Radiachromic® reader. Thus, if it is assumed that the dosimeter temperature during read-out is within $\pm 5^\circ\text{C}$ of its value during calibration, $u_{temp-r} = 0.7 \times 5 / \sqrt{3}$ ($\sim 2\%$) where, $0.7\% ^\circ\text{C}^{-1}$ is the read-out temperature coefficient as estimated in the IAEA Laboratory. The factor of $\sqrt{3}$ is based on the assumption that the dosimeter temperature has rectangular probability distribution within the two limits (ISO 1993). If the read head temperature reported by the DoseReader2® is used to correct the reading this uncertainty becomes insignificant and may be omitted.

Total Uncertainty in the Measured Dose Value, u_{total}

The total uncertainty in the measured dose value, u_{total} (%) is then given by adding these components in quadrature:

$$u_{total} = (u_{dr}^2 + u_{fit}^2 + u_{lot}^2 + u_{temp-i}^2 + u_{temp-r}^2)^{1/2}$$

All these values of u are for 1 standard deviation (σ). However, to imply a higher level of confidence that the ‘true’ value lies within the reported range, u_{total} should be multiplied by a coverage factor k . As a general practice, $k = 2$ is used. Thus, one can state with about 95% confidence that the ‘true’ dose value lays within $D_{measured} \pm 2u_{total}$.

This value has an impact on the dose selected for processing the insects. The target dose that the process should be set for is then calculated as:

$$\text{Target dose} = \text{Minimum dose} + (2 \times u_{\text{total}})$$

4.5. Relevant Literature

Ashland Inc. 2012. Gafchromic® Dosimetry Media, Type HD-V2. <http://www.ashland.com/Ashland/Static/Documents/ASI/Advanced%20Materials/gafchromic-hdv2.pdf>. Accessed 2013-11-17.

ASTM. 2008. E178 - 08 Standard Practice for Dealing with Outlying Observations. ASTM International, West Conshohocken, PA. DOI: 10.1520/E0178-08.

ASTM. 2009. E666 - 09 Standard Practice for Calculating Absorbed Dose from Gamma or X Radiation. ASTM International, West Conshohocken, PA. DOI: 10.1520/E0666-09.

ASTM. 2010. E170 - 10 Standard Terminology Relating to Radiation Measurements and Dosimetry 2010. ASTM International, West Conshohocken, PA. DOI: 10.1520/E0170-10.

ASTM 2013. E1026-13 Practice for Using the Fricke Reference-Standard Dosimetry System. ASTM International, West Conshohocken, PA. DOI: 10.1520/E1026-13.

ASTM. 2013. E275 – 08(2013) Describing and Measuring Performance of Ultraviolet and Visible Spectrophotometers. ASTM International, West Conshohocken, PA. DOI: 10.1520/E0275-08R13.

FAO/IAEA, 2008. Spreadsheet for Dosimetry Calculations, v1.3a [xls]. <http://www-naweb.iaea.org/nafa/ipc/public/ipc-gafchromic-dosimetry-sterile-insect-technique.html>.

FAO/IAEA, 2013a. Dosimetry for SIT: Standard Operating Procedure for Gafchromic® film dosimetry system for gamma radiation, v 2.0, International Atomic Energy Agency, Vienna, Austria. <http://www-naweb.iaea.org/nafa/ipc/public/ipc-gafchromic-dosimetry-sterile-insect-technique.html>.

FAO/IAEA. 2013b. Dosimetry for SIT: Standard Operating Procedure for Gafchromic® film dosimetry system for low energy X radiation. V 1.0, 48pp.

International Organization for Standardization (ISO). 1993. Guide to the expression of uncertainty in measurement, Geneva, Switzerland.

International Organization for Standardization (ISO). 2006. Sterilization of health care products – Requirements for validation and routine control - Radiation sterilization, ISO 11137, Geneva, Switzerland.

ISO/ASTM. 2005. Standard 51431:2005. Practice for Dosimetry in Electron Beam and X-Ray (Bremsstrahlung) Irradiation Facilities for Food Processing. International Organization for Standardization, Geneva. http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=39913.

ISO/ASTM. 2005. Standard 51608:2005. Practice for Dosimetry in an X-Ray (Bremsstrahlung) Facility for Radiation Processing. International Organization for Standardization, Geneva. http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=39914.

ISO/ASTM. 2013. Standard 51261:2013. Practice for Calibration of Routine Dosimetry Systems for Radiation Processing. International Organization for Standardization, Geneva, 2010. http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=60211.

ISO/ASTM. 2013. Standard 51539:2013. Guide for Use of Radiation-Sensitive Indicators. International Organization for Standardization, Geneva. http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=63642.

ISO/ASTM. 2103. Standard 51940:2013. Guide for Dosimetry for Sterile Insect Release Programs. International Organization for Standardization, Geneva. http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=61087.

ISO/ASTM. 2013. Standard 51275:2013. Practice for Use of a Radiochromic Film Dosimetry System. International Organization for Standardization, Geneva,. http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=62954.

Mehta K. and A.G. Parker. 2011. Characterization and dosimetry of a practical x-ray alternative to self-shielded gamma irradiators. Radiat. Phys. Chem. 80: 107-113

Sharpe, P.H.G. 1999. Calibration and traceability in high dose dosimetry, Invited paper at International Symposium on Techniques for High Dose Dosimetry in Industry, Agriculture and Medicine, TECDOC-1070, 281-288. IAEA. Vienna, Austria.

Tuli, J.K. 1994. Nuclear data sheets update for A = 137, Nuclear data sheets, Vol. 72, 136.

Unterweger, M.P., D.D. Hoppes and F.J. Shima. 1992. New and revised half-life measurement results. Nuclear Instruments and Methods, Physics A 312: 349-352.

Wagner J.K. J.A Dillon, E.K. Blythe, and J.R Ford. 2009. Dose characterization of the rad source™ 2400 X-ray irradiator for oyster pasteurization. Appl. Radiat. Isot. 67: 334-339.

5. 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.

5.1. 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 5.1** 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 5.1 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 5.1, b, c**).



Figure 5.1: 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 Mex, 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 5.2**. 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 5.2**).



Figure 5.2: 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 5.3**). As noted below (see 5.2: Shipping and Handling Procedures), the boxes should not be held at temperatures below 18-20°C.

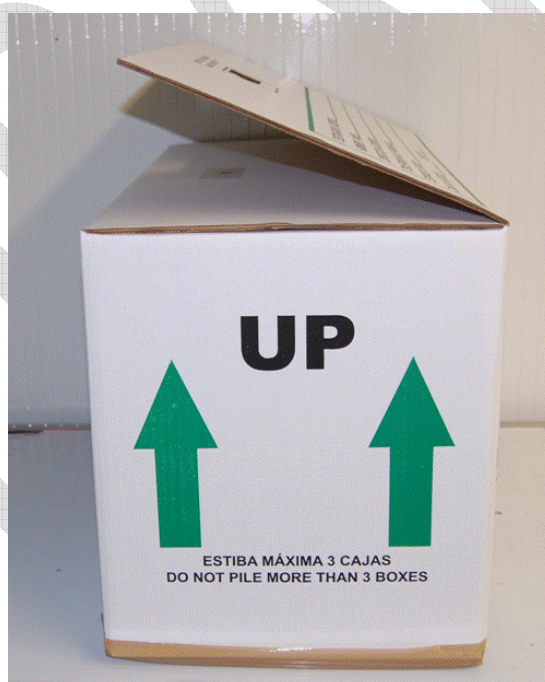


Figure 5.3: Labels placed on boxes containing sterile mexfly pupae shipped from Tapachula (Moscafrut rearing facility) within Mexico, or to California USA.

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”.

5.2. 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 5.4** we can be observe temperature variations (both, inside and outside boxes) in some shipments from Guatemala to Florida USA.

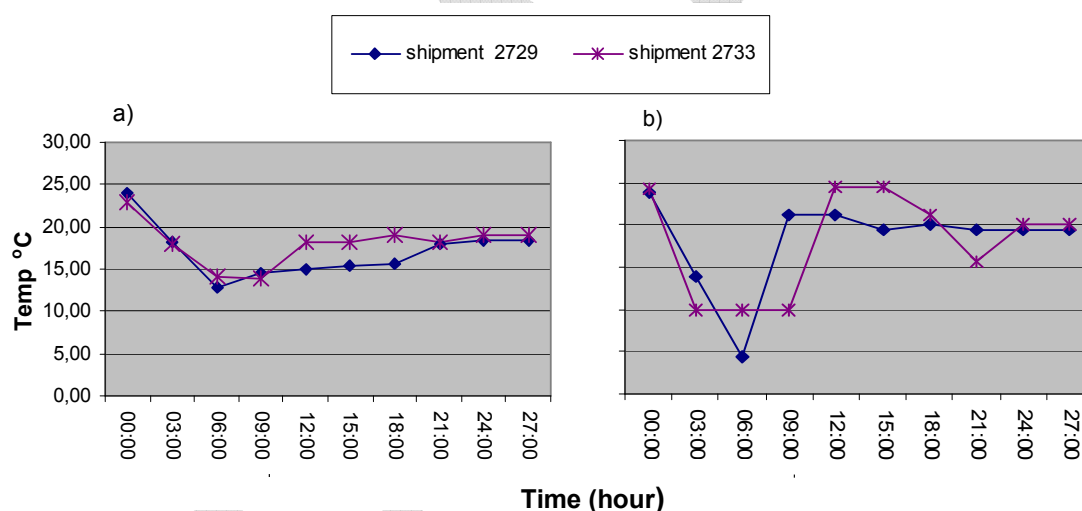


Figure 5.4 Temperature record in two shipments of sterile male of *C. capitata* from Guatemala to Florida: a) inside the box, b) outside the box.

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 transshipment 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 minimum information that the

datasheet should contain is shown in 9.5. Datasheet for Shipment of Sterile Pupae. 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 5.3 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.

5.3. 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 5.5**).
- Documents should include clear instructions to handlers and officials at the point of embarkment, transshipment 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 as shown in **9.5. Datasheet for Shipment of Sterile Pupae**.
- Documents should also include clear instructions to officials at transshipment or entry points on how a lost package that is found is to be discarded.



Figure 5.5: “Transit” documents for shipment of sterile mexfly pupae from Tapachula Mexico to the north of the country or California USA.

5.4. Relevant Literature

Brazzel, J.R., C. Calkins, D.L. Chambers and D.B. Gates. 1986. Required quality control tests, quality specifications, and shipping procedures for laboratory produced Mediterranean fruit flies for sterile insect control programs. APHIS 81-51, USDA-APHIS, Hyattsville, MD, USA.

Enkerlin, W.R and M.M. Quinlan. 2004. Development of an International Standard to facilitate the Transboundary Shipment of Sterile Insects, pp 203-212, IN: Barnes, B.N. (Ed.) Proceedings of the 6th International Symposium on Fruit Flies of Economic Importance. Isteg Scientific Pubs. Irene, South Africa.

FAO/IAEA. 2000. DA.10.16 Gafchromic® Dosimetry System for SIT, Standard Operating Procedures, IAEA, Vienna, Austria, 42 pp.

FAO/IAEA. 2001. Discussion Paper on Transboundary Shipment of Sterile Insects. Prepared by an FAO/IAEA Consultants Group; 30 July to 3 August 2001, Vienna, Austria. 28pp.

FAO/IAEA. 2007. Guidance for Packing, Shipping, Holding and Release of Sterile Flies in Area-Wide Fruit Fly Control Programmes (ed. by W. Enkerlin), Rome, Italy, 134 pp.

Heneberry, T.J. 1983. Considerations in sterile insect release methodology. USDA-ARS.

International Plant Protection Convention (IPPC). 2012. Glossary of Phytosanitary Terms. ISPM Pub. No.5, FAO, Rome.

Planta Moscafrut SAGARPA-IICA. 2009. Manual de Envíos del Material Biológico Producido en la Planta Moscafrut. Metapa de Domínguez Chiapas, México, 30 pp.

Zavala, J.L., M.M. Fierro, A.J. Schwarz, D.H. Orozco and M. Guerra. 1985. Dosimetry practice for the irradiation of the Mediterranean fruit fly *Ceratitis capitata* (Wied.). 23-30. In IAEA [ed.], High dose dosimetry, Proceedings of the International Symposium, STI/PUB/671. IAEA. Vienna, Austria.

6. 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 predicted feral population control.

In order 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 proposed. These tests document, at each step of the process, the quality of SI before and after each of the handling phases that occur within 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 determining if there are losses in SI quality, at which phase of the process they are occurring, and in turn, this would allow taking 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.

6.1. Guidelines for Sampling Insects for Routine QC Tests

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

6.2. Quality Control Tests on Arrival

Evaluation of SI shipments at the time of 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 receiving end are therefore important. Among the factors that need to be ensured for comparison purposes is the calibration of measuring equipment

(Metrology) the similarity of test procedures as well as the timing of these measurements (i.e. insects of similar age).

In order to compare if detriments in quality have been introduced during the shipping procedures, a set of tests similar to those that the rearing facility conducts prior to the shipment departure is mandatory. The comparative evaluations of insect quality are represented by the following procedures/tests:

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

6.2.1. 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.

6.2.2. Pupal weight

Objective

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

Pupae weight, as determined by the fly emergence and release facilities at arrival, is a direct comparison with the value determined when the pupa leaves the rearing facility. It has been documented that pupae weight is gradually reduced as it approaches the emergence of the adult (Langley 1970; Nestel et al. 2003).

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. The importance of this measurement is that the value determined, is also an indirect measurement of longevity. This is of particular importance for fly emergence and release facilities due to the expense incurred in sterile insect's releases and the need to ensure insect quality when conducting field releases. Low pupae weights can be the result of low quality rearing procedures and as such have to be reported to production managers to ensure modification in their rearing practices.

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).

6.2.3. Emergence and flight ability

Objective

To obtain an accurate estimate of the percentage of pupae, at time of their arrival at fly emergence and release facilities, that will emerge and produce flies that are able to fly.

Discussion

Fly emergence and release facilities receive pupal shipments that originate at different distances from where the actual 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 flies that are able to fly at the time of their arrival. The results of these evaluations should be compared to the results obtained at the rearing facility prior shipment (i.e., test post-irradiation).

Equipment and test conditions

To obtain estimates of the percentage of emergence and percentage of flies that are able to fly of sterile insects the same equipment and testing conditions as those already suggested in section 2.2 of this manual apply (see 2.2.2. Emergence and Flight Ability).

Procedures

The main difference between the methodology already described on how to perform these tests at the rearing facilities and the fly emergence and release facilities is that the age of pupae at arrival is uncertain (as it has been affected by shipping temperatures and hypoxia). Fly emergence and release facilities at arrival of the pupae shipment should therefore open the containers that transport the pupae (break hypoxia) and immediately prepare the arrived pupae for testing.

Interpretation

Appropriate percentage of emergence and percentage of flies that are able to fly values reflect that the conditions for shipping SI to the fly emergence and release facilities have been adequate. It has been documented that a long period of hypoxia could have a detrimental effect on these values, reducing them as the numbers of hours of hypoxia increase (FAO/IAEA, 2007). Lower relative humidity during the rearing process at fly emergence and release facilities can have an effect on lower percentage of emergence and percentage of flies that are able to fly. Comparisons conducted between the mass-rearing facilities and fly emergence and release facilities are important to determine if the conditions for appropriate percentage of emergence and percentage of flies that are able to fly were met. A compilation for the different fruit fly species of what is currently considered an appropriate percentage of emergence and percentage of flies that are able to fly presented in **Table 2.2**, see 2.2.2 Emergence and Flight Ability.

6.2.4. Survival under stress

Objective

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

Discussion

This test is a relative measure of the nutrient reserves available to the adult fly at the time of emergence. This measurement can be compared to the survival of the sterile insects as determined by the mass-rearing facility prior to their departure to the fly emergence and release facilities. This comparison also allows determining the robustness of packing and shipping procedures.

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

Procedures

This test should be conducted as described in section 2.2.3 Survival under Stress, without providing food and/or water to the adults. The length of time considered for this test varies according to the fruit fly species and its agreed requirement is presented in **Table 2.3**, in section 2.2.3 Survival under Stress of this manual. The results obtained in this evaluation need to be reported as percent survival.

6.2.5. 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

At 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 efforts. For species like Mediterranean fruit fly in which genetic sexing strains (GSS) are being used, the expected sex ratio is skewed toward the males. Nevertheless, ratios should be determined since the presence of small amounts of females can be misleading in scenarios like the preventative release programs in California and Florida.

The age of the pupae at the time of arrival at the fly emergence and release facilities is related to its emergence time, but there are factors besides pupae age that could affect emergence time (e.g. time in hypoxia, shipment conditions, or temperature during their fly emergence and release facility handling process). Determining the time of emergence of SI is important because it also allows calculating the approximate age of the sterile insects at the release time. This age should be as much as possible close 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 have already been described in section 2.3.4. Sex Ratio and Timing of Emergence.

Interpretation

In addition to what has already been stated in section 2.3.4. Sex Ratio and Timing of Emergence. The fly emergence and release facilities location and the environmental conditions of the surrounding area may also play a role in the conditions inside the rearing facility. The fly emergence and release facilities managers should be aware that the appropriate environmental conditions during the rearing operation would allow adequate emergence time and sexual maturation of insects prior to their release.

6.3. Quality Control Post Handling/Chilling

6.3.1. Absolute 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 an average temperature of 3.3°C (38 °F). 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 40 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 absolute 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 5 ml plastic beakers randomly collect 5 samples of 5 ml chilled sterile insects 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 and 65% RH. After a period of four hours (see **Figure 6.1**), flight tubes are covered with a lid and are transferred/placed inside a freezer. Covered tubes are placed inside a freezer for a minimum of 3 hours to ensure all insects are dead before the weight of the remaining insects in the tubes is determined. These times need to be adjusted according to the fly species.

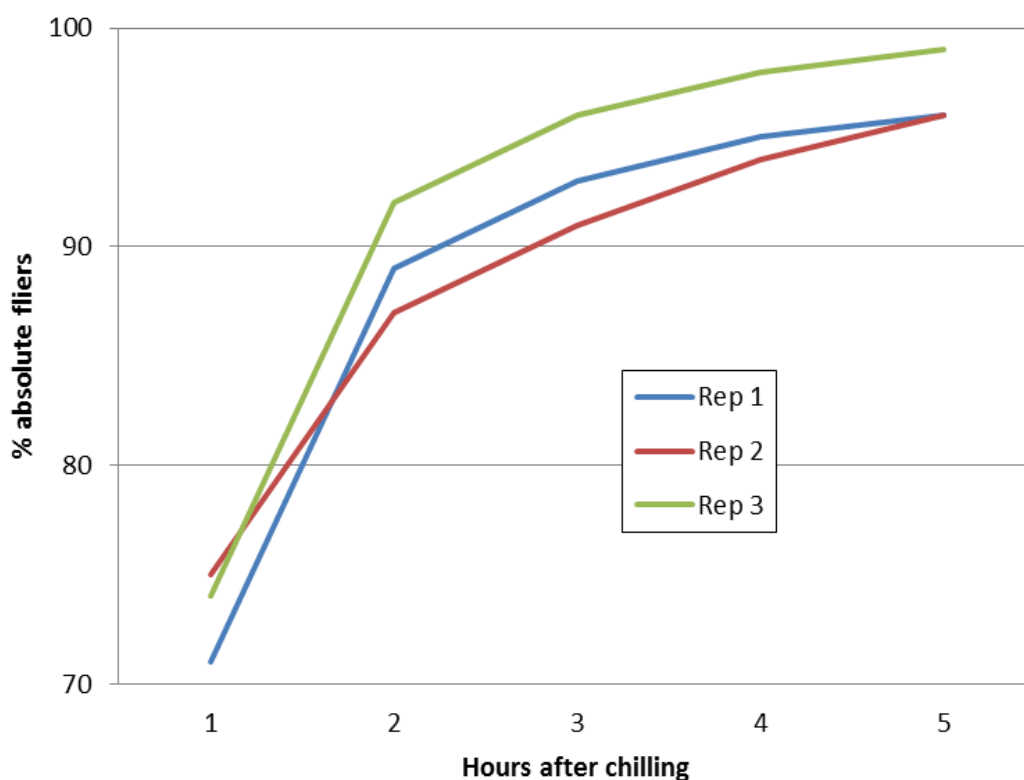


Figure 6.1 Cumulative percentage of *Ceratitis capitata* flies out of the tube (absolute fliers) five hours after chilling.

Absolute fliers are those adult flies in the sample that escape from the container flying. The value of this parameter is represented by the difference between the total fliers in the sample and the non-fliers that remained inside flight tubes. The percentage of absolute fliers (AF) is calculated as follows:

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

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.

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 SI 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 an 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 insects viability. For users of bag releases, the crowding conditions during transportation can have a severe effect in reducing flight ability; operational practices should be validated/questioned prior to full implementation.

6.3.2. 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.

Discussion

The objective, general 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 samples from the chilled adult release box, or from bags or boxes that are placed under cold temperatures to be able to handle the flies for the test.

Furthermore this test should be run weekly both without food and water (see **Table 2.3**, section 2.2.3) and also without food and with water.

Interpretation

In addition to what has been stated in section 2.2.3: Survival under Stress, the survival of sterile insects during the fly emergence and release facilities rearing and handling process can be optimized by providing insects with good quality and quantity "adult food". Issues like accessibility of insect to food sources as well as the crowding conditions within the rearing 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.

6.4. 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 6.3.1 Absolute 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.

6.4.1. Absolute fliers

Objective

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

Discussion

Sterile insects are exposed to cold temperatures in order to immobilize them to facilitate their handling and eventual field distribution. Chilled insects 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 absolute fliers post- release needs to be determined.

Equipment

Same equipment as used for Absolute fliers post-chilling (section 6.3.1 Absolute 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 6.2b**).

Procedure

During the knockdown process in cold rooms, 5 samples of 100 chilled flies each are taken randomly and are carefully introduced into 5 velcro-net bags (**Figure 6.2b**). 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 6.2a**). 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 6.2: (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

Absolute is determined by the difference between the total fliers in the sample and the non-fliers that remained inside. Percentage of absolute fliers is calculated according to the formula showed in section 6.3.1 Absolute fliers.

6.4.2. Survival chilled/released flies under stress

Objective

Determine survival of sterile insects after their field releases.

Discussion

The determination of adult fly survival after chilling is a relevant test to predict how successful the SIT could potentially be if sterile insects survive well after the handling and chilling process. This test should be conducted with water but without food to simulate the field conditions faced by the flies after releases. This measurement should be established as an indicator of the effect of the chilling process and the ferry time to the release site on the overall insect quality. This test serves also as an estimate of nutrient reserves of the sterile flies. This test may also provide means of estimating the quality of the adult diets provided in the fly emergence and release facilities.

Equipment and Procedures

The general equipment needs and testing procedures are similar to those described in the methodology in section 6.3.2 Survival chilled/released flies under stress.

To perform this test the samples must be taken at the airport from the release machine when the plane returns from the aerial release. Test frequency recommended is two times per week.

Interpretation

Every 12 h the number of dead fly must be registered. The test should be extended to the time required to exceed 50% mortality. Survival is expressed as the number of accumulated hours in which 50% of flies survive. This final determination of sterile insects survival is relevant to

the operational activities that may be required in order to release the required numbers of sterile insects in the field (i.e. programming field releases).

6.5. Relevant Literature

FAO/IAEA. 2007. Guidance for Packing, Shipping, Holding and Release of Sterile Flies in Area-Wide Fruit Fly Control Programmes (ed. by W. Enkerlin), Rome, 134 pp.

Langley, P. A. 1970. Physiology of the Mediterranean fruit fly in relation to the sterile-male technique, pp. 25-32. *In*: Anon. (ed.), Physiology of the Mediterranean fruit fly in relation to the sterile-male technique. Proceedings of a panel held in Vienna in 1969. International Atomic Energy Agency, Vienna, Austria.

Manual de Control Autocida de la Mosca del Mediterraneo Esteril por el Sistema de Adulto Frío, 2004. Pruebas de Control de Calidad de la Mosca del Mediterráneo Estéril, por la Técnica de Adulto Frío. Guatemala, 58p.

Nestel, D., D. Tolmasky, A. Rabossi, and L.A. Quesada-Allué. 2003. Lipid, carbohydrates and protein patterns during metamorphosis of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae). *Ann. Entomol. Soc. Am.* 96(3): 237-244.

Planta Moscafrut. 2010. Manual de Control de Calidad de Moscas de la Fruta Estériles y Parasitoides (procedimientos para evaluar el Producto en Centros de Empaque), Dirección Moscas de la Fruta DGSV-SENASICA-SAGARPA, Mexico, 63p.

USDA/APHIS. 2008. United States, Mexico and Guatemala Fruit Fly Emergence and Release Facilities Review, 79 p.

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

7.1. 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

- Irradiator (gamma or X ray).
- 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; filter paper and sponge moistened with water.
- Incubator or other environmentally controlled space.
- Dissecting microscope and equipment
- Fine paintbrushes.

Test Conditions

- Temperature $25 \pm 1^{\circ} \text{C}$
- Humidity $65 \pm 15\% \text{ RH}$
- 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 are irradiated according to standard procedures (see chapter 3 Irradiation). This will help ensure that dosages absorbed by the test insects are comparable to those absorbed by insects that are destined for release in the field. After irradiation, a random sample of pupae should be taken ensuring that pupae from all positions in the container are represented.

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)

The sterility test is simplified for genetic sexing strains, such as Mediterranean fruit fly, where only males are produced for release. In this case, 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 7.1**).



Figure 7.1: 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. A sample recording sheet is provided in section 9.6 Sterility Assessment Form.

For routine tests of sterility a minimum of 500 eggs to be collected is recommended. 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. High levels of sterility (irradiated males x non-irradiated females producing <0.5% hatch) are required when sterile *C. capitata* are released into areas that are historically free of the pest. 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.

7.2. 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.

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. This is done by releasing sterile mass-reared males (and females in cases of bisexual strains) and analysing the respective number of matings achieved by wild and laboratory males with wild females.

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. This is done by releasing males and females from two different origins and analysing the number of mating couples obtained in each mating combination.

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. This is done by slightly modifying the general test protocol to assess:

- 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,
- 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 8).

Discussion

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 laboratory- 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 three groups of scientists who jointly participated in three 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) and one on improving sterile male performance (2004-2009) 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 sexual compatibility and male sexual competitiveness 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 for mating performance field cage test are collected from the field as eggs or larvae in fruit. Typically, the main host fruit available in the region and during the season of the tests is selected as a source of wild flies. Infested fruits of the selected host should be collected directly from host plants not treated with insecticides and returned to the laboratory. Fruit are placed on wide mesh trays or racks over a pupation substrate such as lightly moistened sand or vermiculite. The substrate is then gently sifted every 2-3 days and wild pupae are collected. Given that fruit holding methods vary widely with the fly species, fruit, and local conditions, emphasis should be placed in providing the appropriate conditions to avoid larvae and pupae mortality. Pupae are then placed in a screen or Plexiglas cage used for "Emergence and Flight

Ability Tests”, providing water and food. Food should resemble what flies obtain from nature, being one possibility an open fig. Dried commercial figs, or other fruit, can be re-hydrated and placed as a nutrients source. Within a few hours of adult emergence, select only flying adults and separate the sexes. Place the flies in laboratory cages (screen or Plexiglas) with a density of 30 ± 10 flies per litre volume for the case of medfly 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 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. Hold the flies according to the day of emergence with water and food until sexually mature. Both wild and laboratory adults find and feed on protein sources in nature and for this reason some protein should be added in the food. However, as already stated above, it is recommended that this source resembles as much as possible as what can be found in nature. Recent studies are showing that several species are able to balance their nutrient intake from different food sources so it is desirable then 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. 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 7.1**). 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.

Table 7.1: Recommended fly’s age (days) at which to perform field cage tests according to the fruit fly species.

Species	Wild flies	Laboratory flies
<i>Ceratitidis capitata</i>	7-10	4-6
<i>Anastrepha ludens</i>	16- 21	10
<i>Anastrepha obliqua</i>	15-17	8-10
<i>Anastrepha fraterculus</i>	15-20	10-15
<i>Bactrocera cucurbitae</i>	30	10
<i>Bactrocera dorsalis</i>	25-30	20
<i>Bactrocera tryoni</i>	20	10

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 (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 facility, 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 7.1**) 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.

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.

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 Mediterranean fruit fly sterile males 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 7.2**). 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 7.2: 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 gr per kilo 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 7.3**).

For the case of adding the colorant to the diet, it is advisable to do it since adult emergence. A small drop of 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 7.4**.

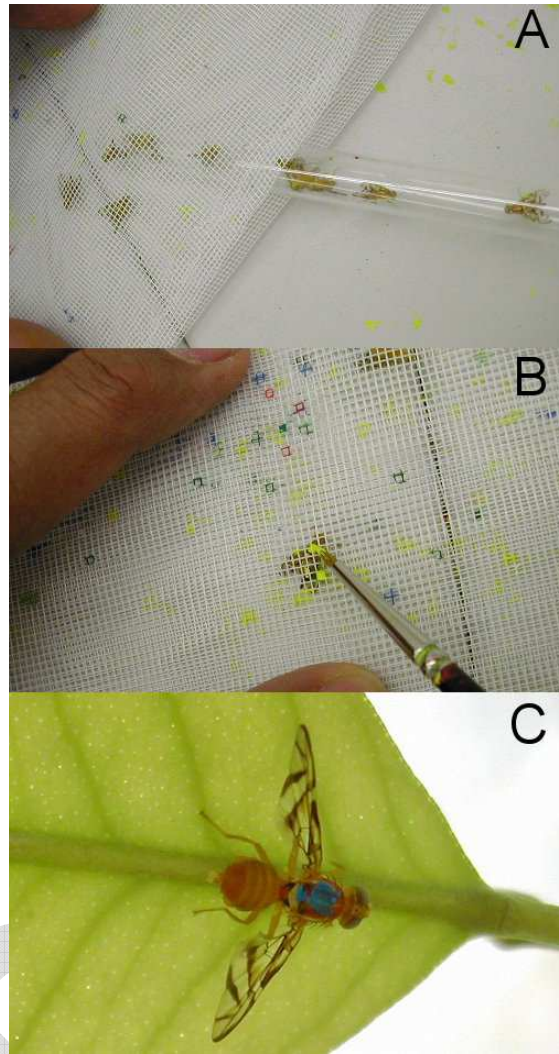


Figure 7.3: Procedure for marking tephritid adult individuals with water-based paint. (A) adults are gently blown from the aspirator into a bag; (B) the bag is stretched to immobilize the fly and a drop of paint is made on the notum; (C) the result: a marked *Anastrepha fraterculus* male calling in a field cage.



Figure 7.4: 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 7.5**).

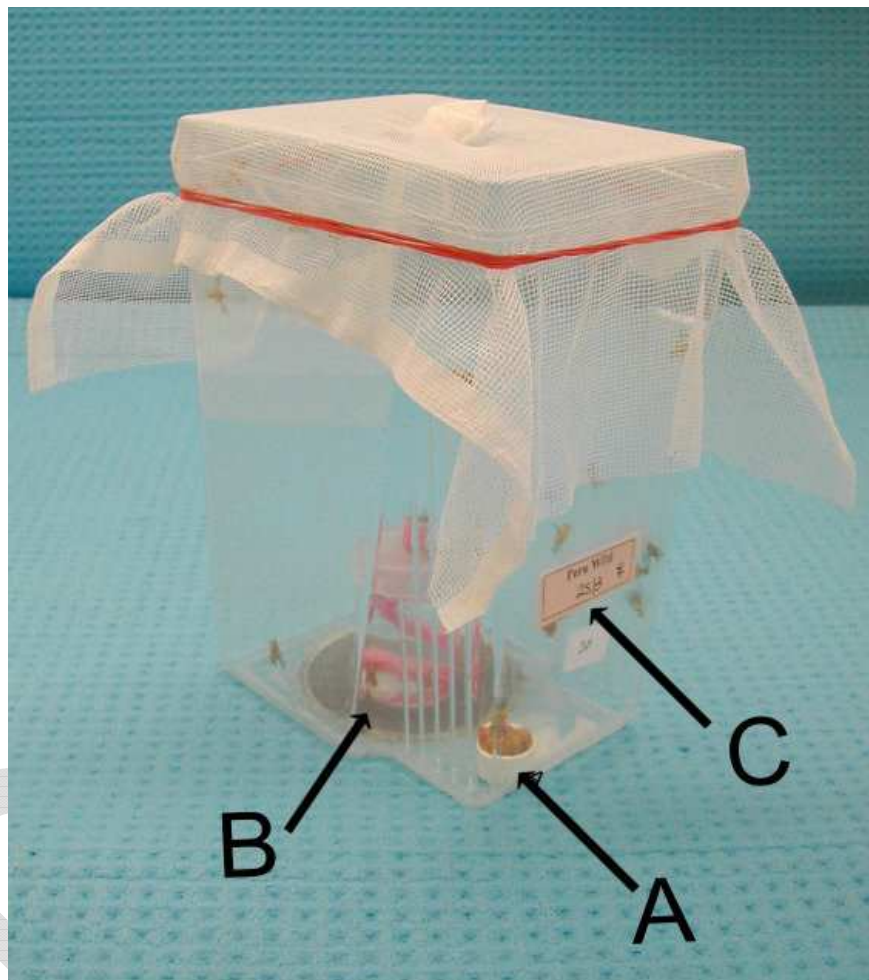


Figure 7.5: Container used to hold the flies after individual marking and before releasing into the field cage. A: food; B: water; C: label mentioning the strain, sex and number of adults.

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 7.6**). 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 7.6: 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 χ^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 include three replicates of three different batches of sterile flies and three different days, with at least 9 cages being evaluated (three replicates of each batch and three days to evaluate the three different batches). 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 7.7**). 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.

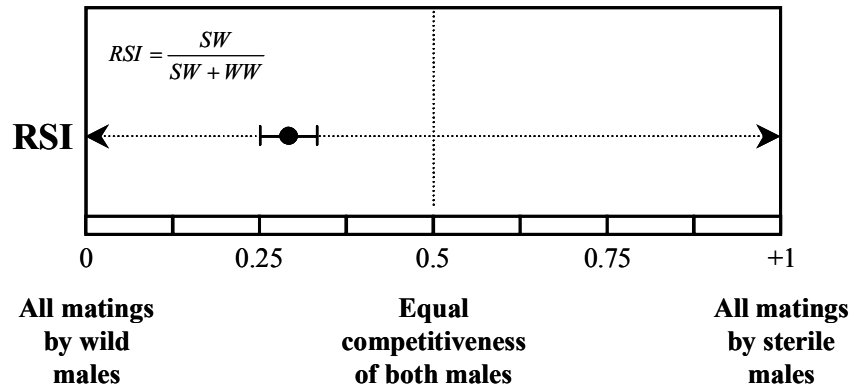


Figure 7.7: Graphic representation of the Relative Sterility Index (RSI). The value shown (mean \pm se) was obtained when comparing wild and sterile *Anastrepha ludens* flies in field cages (after Hernandez et al. 2003).

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 7.8**).

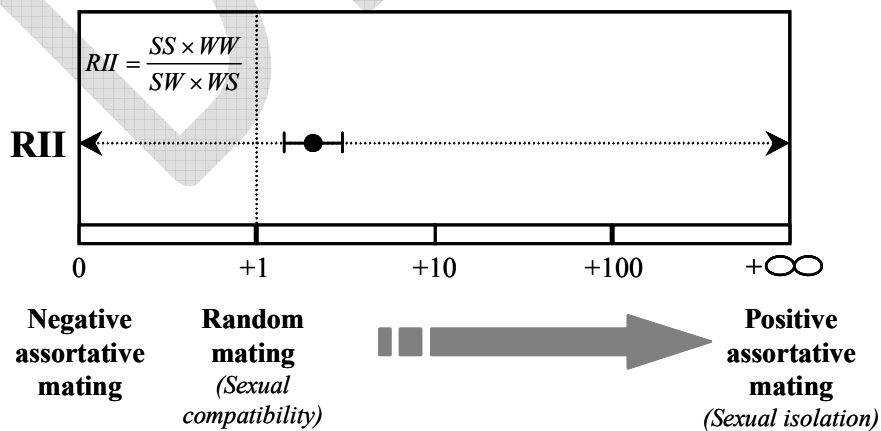


Figure 7.8: Graphic representation of the Relative Isolation Index (RII). The value shown (mean \pm se) was obtained when comparing wild and sterile *Ceratitis capitata* flies in field cages.

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 7.9**). 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.

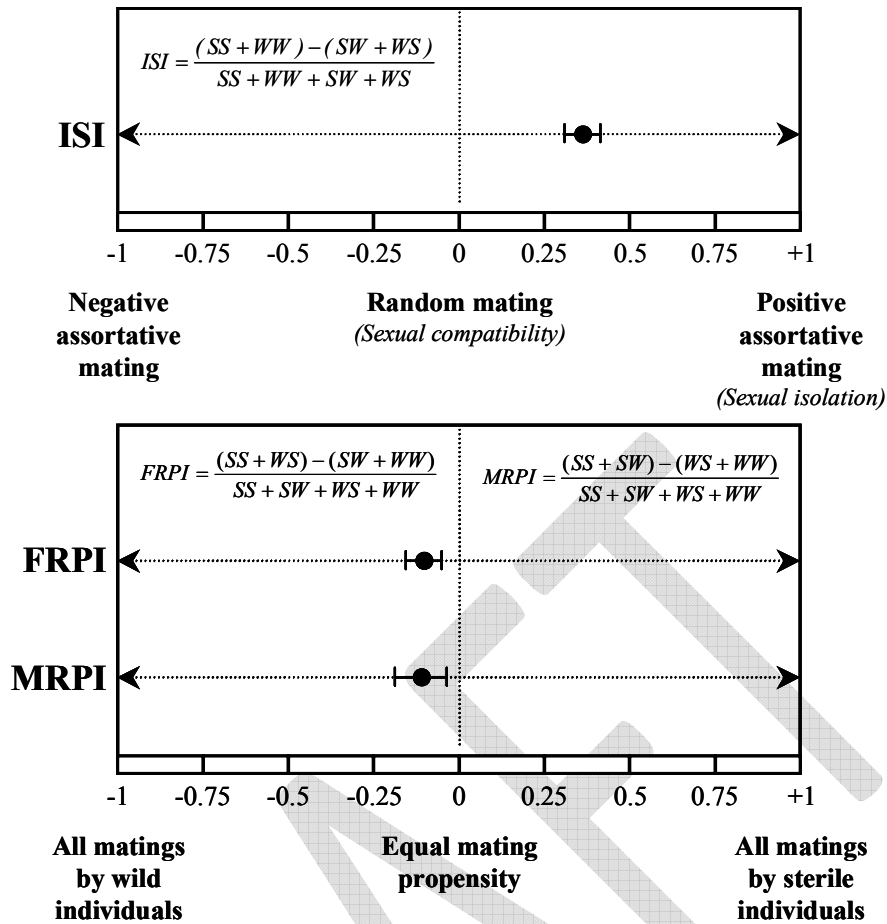


Figure 7.9: Graphic representation of the Isolation Index (ISI) and of the Male and Female Relative Performance indices (MRPI and FRPI). These indices should be considered together for a better understanding. The value shown (mean \pm se) was obtained when comparing wild and sterile *Anastrepha ludens* flies in field cages (after Hernandez et al. 2003).

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.

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 7.9**). 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 below 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 7.9**).

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

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:

$$\text{Latency} = \text{Copula start time} - \text{Time of onset of field cage test}$$

Mean values are computed separately for each cage and comparisons between mating types can be analysed by means of ANOVA or t-tests.

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 ANOVA or t-tests.

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 can 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 collected (usually the tree 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, stem) 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.

7.3. 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.

Discussion

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. To do this, different released areas are selected and in a given date, flies are released with a different colour than the routinely used. Traps are serviced at different times in each of the releasing areas. In the following releasing dates, the time between release and trap servicing is changed in the different release areas and this procedure is done on a regular basis. Data gathered from the trap captures is used to estimate survival as proposed in the section 7.5 Release-Recapture Test of Dispersal and Survival.

Procedures

Select three to four areas that are subjected to sterile male releases in the operational programme. Coordinate the releasing and trap servicing schedules in a way that in one of each of the areas the flies are released 1, 3, 5 and 7 days before the traps are serviced. Ensure that at these releasing dates, released flies have been painted with a different colour than the routinely used. Repeat this procedure at a regular basis (once a month) rotating the time between fly release and trap servicing in each area. Recover the data of the amount of flies recovered in each area from the trapping service.

Other option to perform this test is to select one area, change the colour of the released flies and revise the traps at different dates (e.g. at days 1, 3, 5 and 7 after release). In any case, the idea is to modify the least the procedures of the normal release but to maximize the most the information and conclusions derived from the data obtained. Data gathered from the Survival in Field Cages Tests (see Ancillary Tests, section 8.9) can be run in parallel to this test in order to compare results.

Interpretation

Estimate survival as in the Release-Recapture Test of Dispersal and Survival (section 7.5), by plotting the number of capture flies at each releasing day. If sufficient replicates are done, any bias originated from some particular areas being harsh or adequate for flies' survival will compensate any under or over estimate of survival. Data 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. 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).

7.4. 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.

Discussion

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 predators 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 design to be conducted under laboratory conditions and the flies are confined to small containers. More recently, 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.

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 as eggs or larvae in fruit following the procedures described in section 7.2 Mating Performance Field Cage Test. Before adult emergence, pupae should be painted with the fluorescent dye.

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 7.2 Mating Performance Field Cage Test. Pupa should be painted with the fluorescent dye using a different colour of that of the wild flies. In this case 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 7.2 Mating Performance Field Cage Test). 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 with fluorescent powder.
- 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 as described in section 7.2 Mating Performance Field Cage Test.
- 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.

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. Carry on the test 9 times try to cover at least three different batches of mass-reared flies and three different days. 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 the desired number of predators are released in the cage and left 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.

Interpretation

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. 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).

7.5. Release-Recapture Test of Dispersal and Survival

Objective

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

Discussion

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 zone. As 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.

If wild flies are to be released in the cages as well, then flies should be painted with the fluorescent powder taking special care not to use the same colour as the sterile flies. Procedures to hold the flies before release should resemble that of the described in in section 7.2 Mating Performance Field Cage Test.

Equipment

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

- Screen cages, small PVC pipe framed cages as in the Survival in Field Cages Test (in Ancillary Tests, see Figure 8.3).
- Miscellaneous cups, forceps, or other supplies as necessary.

Procedures

As mentioned before, sterile dye-marked insects are shipped to the release facility and handled according to the standard releasing protocol.

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 (e.g., 10,000 per strain or more) must be released in order to obtain meaningful data. Numbers of pupae for release may be determined volumetrically, by weight, or using a calibrated optical seed counter. Actual numbers of flies released is then estimated by sampling the pupae that remain after the release to determine emergence rate.

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.

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.
- 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 the 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 (\bar{d}) from release site to trap:

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

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.

7.6. Relevant Literature

Allinghi, A., G. Calcagno, N. Petit-Marty, P. Gómez Cendra, D. Segura, T. Vera, J. Cladera, C. Gramajo, E. Willink and J.C. Vilardi. 2007. Compatibility and Competitiveness of a Laboratory Strain of *Anastrepha fraterculus* (Diptera: Tephritidae) after Irradiation Treatment. Fla. Entomol. 90(1): 27-32.

Aluja, M., I. Jacome, A. Birke, N. Lozada and G. Quintero. 1993. Basic patterns of behavior in wild *Anastrepha striata* (Diptera: Tephritidae) flies under field cage conditions. Ann. Entomol. Soc. Am. 86(6): 776-793.

Baker, P.S. and A.S.T. Chan. 1991. Identification of tephritid fruit fly dispersal. Guidelines for a sterile release programme. J. Appl. Entomol. 112: 410-421.

Baker, P.S., A.S.T. Chan and M.A. Jimeno-Zavala. 1986. Dispersal and orientation of sterile *Ceratitidis capitata* and *Anastrepha ludens* (Tephritidae) in Chiapas. J. Appl. Ecol. 23: 27-38.

Bateman, A. J. 1949. Analysis of data on sexual isolation. Evolution. 3: 174-177.

Bloem, K., S. Bloem, D. Chambers and E. Muñiz. 1993. Field evaluation of quality: release-recapture of sterile medflies of different sizes. 295-296 In Aluja, M., and P. Liedo [eds.] Fruit flies: biology and management. Springer-Verlag, New York, NY, USA.

Boller, E.F., U. Remund, B.I. Katsoyannos and W. Berchtold. 1977. Quality control in European cherry fruit fly: evaluation of mating activity in laboratory and field cage tests. Z. Angew. Ent. 83: 183-201.

Boller, E.F., B.I. Katsoyannos, U. Remunf and D.L. Chambers. 1981. Measuring, monitoring and improving the quality of mass-reared Mediterranean fruit flies, *Ceratitidis capitata* (Wied.) 1. The RAPID quality control system for early warning. J. Appl. Entomol. 92: 67-83.

Bonizzoni, M., L.M. Gomulski, S. Bertin, F. Scolari, C.R. Guglielmino, B. Yuval, G. Gasperi and A.R. Malacrida. 2007. Unfaithful Mediterranean fruit fly *Ceratitidis capitata* females: impact on the SIT?. 175-182. In Vreysen, M. J. B., A. S. Robinson and J. Hendrichs. Area-wide control of insect pests. From research to field implementation. Springer, Dordrecht, The Netherlands.

- Briceño, R.D. and W.G. Eberhard. 1998.** Medfly courtship duration: a sexually selected reaction norm changed by crowding. *Ethology Ecology & Evolution*. 10: 369-382.
- Briceño, D., W. Eberhard and T. Shelly. 2007.** Male Courtship Behavior in *Ceratitidis capitata* (Diptera: Tephritidae) that Have Received Aromatherapy with Ginger Root Oil. *Fla. Entomol.* 90(1): 175-179.
- Briceño, R.D., W.G. Eberhard, J.C. Vilardi, P. Liedo and T.E. Shelly. 2002.** Variation in the intermittent buzzing songs of male medflies (Diptera: Tephritidae) associated with geography, mass-rearing, and courtship success. *Fla. Entomol.* 85(1): 32-40.
- Briceño, R.D., D. Ramos and W.G. Eberhard. 1996.** Courtship behavior of male medflies *Ceratitidis capitata* (Diptera: Tephritidae) in captivity. *Fla. Entomol.* 79(2): 130-143.
- Burk, T. and J.C. Webb. 1983.** Effect of male size on calling propensity, song parameters, and mating success in Caribbean fruit flies, *Anastrepha suspensa* (Loew) (Diptera: Tephritidae). *Ann. Entomol. Soc. Am.* 76: 678
- Cáceres, C., D.O. McInnis, T. Shelly, E. Jang, A. Robinson and J. Hendrichs. 2007.** Quality management systems for fruit fly (Diptera: Tephritidae) sterile insect technique. *Fla. Entomol.* 90(1): 1-9.
- Calcagno, G.E., F. Manso and J.C. Vilardi. 2002.** Comparison of mating performance of medfly (Diptera, Tephritidae) genetic sexing and wild type strains: field cage and video recording experiments. *Fla. Entomol.* 85(1): 41-50.
- Calkins, C.O. and J.C. Webb. 1983.** A cage and support framework for behavioral tests of fruit flies in the field. *Fla. Entomol.* 66(4): 512-514.
- Calkins, C.O. and A.G. Parker. 2005.** Sterile Insect Quality. 269-296. In Dyck, V. A., J. Hendrichs and A. S. Robinson. [eds.], *Sterile Insect Technique. Principles and Practice in Area-Wide Integrated Pest Management*. Springer, Dordrecht, The Netherlands.
- Cayol, J.P. 2000.** Changes in sexual behavior and in some life history traits of Tephritid species caused by mass-rearing processes. 843-860. In Aluja, M. and A. Norrbom [eds.], *Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior*. CRC Press. Boca Raton, Florida.
- Cayol, J.P., J. Vilardi, E. Rial and M.T. Vera. 1999.** New indices and method to measure the sexual compatibility and mating performance of medfly (Diptera, Tephritidae) laboratory reared strains under field cage conditions. *J. Econ. Entomol.* 92(1): 140-145.
- Cayol, J.P., P. De Coronado and M. Taher. 2002.** Sexual compatibility in medfly (Diptera: Tephritidae) from different origins. *Fla. Entomol.* 85(1): 51-57.
- Chambers, D.L., C.O. Calkins, E.F. Boller, Y. Itô and R.T. Cunningham. 1983.** Measuring, monitoring and improving the quality of mass-reared Mediterranean fruit flies, *Ceratitidis capitata* (Wied). 2. Field tests for confirming and extending laboratory results. *J. Appl. Ent.* 95: 285-303.
- Collins, S.R., C.W. Wldon and P.W. Taylor. 2009.** Effects of filed cage colour and supplementary shade on environmental conditions and mating behavior of Queensland fruit fly, *Bactrocera tryoni*. *Entomol. Exp. et Appl.* 129: 142-147.
- Dunham, M. 1994.** An easily-constructed temporary cage for studying animals in the field. *Fla. Entomol.* 77(4): 505-508.
- Faria, M.J., R. Pereira, T. Dellinger and P.E.A. Teal. 2010.** Influence of methoprene and protein on survival, maturation and sexual performance of male *Ceratitidis capitata* (Diptera: Tephritidae). *J. Appl. Entomol.* 132: 812-819.

- Gómez Cendra, P., D. Segura, A. Allinghi, J. Cladera and J. Vilardi. 2007.** Comparison of Longevity between a Laboratory Strain and a Natural Population of *Anastrepha fraterculus* (Diptera: Tephritidae) under Field Cage Conditions. Fla. Entomol. 90(1): 147-153.
- Haq, I.U., C. Cáceres, P.E.A. Teal, V. Wornoayporn, C. Stauffer and J. Hendrichs. 2010.** Effects of the juvenile hormone analogue methoprene and dietary protein on male melon fly *Bactrocera cucurbitae* (Diptera: Tephritidae) mating success. J. Insect Physiol. 56: 1503-1509.
- Hendrichs, J. 1986.** Sexual selection in wild and sterile Caribbean fruit flies, *Anastrepha suspensa*. M.Sc.Thesis. Univ. of Florida, Gainesville, Fla. 263 pp.
- Hendrichs, J., B. Katsoyannos, V. Wornoayporn, and M.A. Hendrichs. 1994.** Odour-mediated foraging by yellowjacket wasps (Hymenoptera: Vespidae): predation on leks of pheromone-calling Mediterranean fruit fly males (Diptera: Tephritidae). Oecologia 99:88-94.
- Hendrichs, J., V. Wornoayporn, B. I. Katsoyannos and K. Gaggli. 1993.** First field assessment of the dispersal and survival of mass reared sterile Mediterranean fruit fly males of an embryonal temperature sensitive genetic sexing strain. 453-462. In IAEA [ed.], Management of insect pests: nuclear and related molecular and genetic techniques. IAEA. Vienna, Austria.
- Hendrichs, M.A. and J. Hendrichs. 1998.** Perfumed to be killed: interception of Mediterranean fruit fly (Diptera: Tephritidae) sexual signaling by predatory foraging wasps (Hymenoptera: Vespidae). Ann. Entomol. Soc. Am 91:228-234.
- Hendrichs, M.A., V. Wornoayporn, B. Katsoyannos and J. Hendrichs. 2007.** Quality control Method to measure predator evasion in wild and mass-reared Mediterranean fruit flies (Diptera: Tephritidae). Fla. Entomol. 90(1): 64-70.
- Hernandez, E., D. Orozco, M. Hernandez, J.S. Meza, S. Flores and J. Dominguez. 2003.** Quality assurance of mass produced and released fruit flies: third progress report. RC 10839/R2. IAEA. Vienna, Austria.
- Hernández, E., D. Orozco, S. Flores Breceda and J. Domínguez. 2007.** Dispersal and Longevity of Wild and Mass-reared *Anastrepha ludens* and *Anastrepha obliqua* (Diptera: Tephritidae). Fla. Entomol. 90(1): 123-135.
- Hibino, Y. and O. Iwahashi. 1989.** Mating receptivity of wild type females for wild type males and mass-reared males in the Melon fly, *Dacus cucurbitae* Coquillett (Diptera: Tephritidae). Appl. Ent. Zool. 24(1): 152-154.
- Hibino, Y. and O. Iwahashi. 1991.** Appearance of wild females unreceptive to sterilized males on Okinawa Island in the eradication program of the Melon fly, *Dacus cucurbitae* Coquillett (Diptera: Tephritidae). Appl. Ent. Zool. 26(2): 265-270.
- (IAEA) International Atomic Energy Agency. 2003.** Trapping guidelines for area-wide fruit fly programmes. Joint FAO/IAEA Division. Vienna, Austria. 47pp.
- Iwahashi, O., Y. Ito and M. Shiyomi. 1983.** A field evaluation of the sexual competitiveness of sterile melon flies, *Dacus (Zeugodacus) cucurbitae*. Ecol. Entomol. 8: 43-48.
- Jackson, C.G. and J.P. Long. 1997.** Mating behavior of *Bactrocera latifrons* (Diptera: Tephritidae) in field cages. Ann. Entomol. Soc. Am. 90(6): 856-860.
- Jang, E.B., D.O. McInnis, D.R. Lance and L.A. Carvalho. 1998.** Mating-induced changes in olfactory-mediated behavior of laboratory-reared normal, sterile, and wild female Mediterranean fruit flies (Diptera: Tephritidae) mated to conspecific males. Ann. Entomol. Soc. Am. 91(1): 139-144.
- Jordão Paranhos, B., N.T. Papadopoulos, D. McInnis, C. Gava, F.S.C. Lopes, R. Morelli and A. Malavasi. 2010.** Field dispersal and survival of sterile medfly males aromatically treated with ginger root oil. Environ. Entomol. 39(2): 570-575.

- Katsoyannos, B.I., N.T. Papadopoulos, J. Hendrichs and V. Wornoayporn. 1989.** Comparative response to citrus foliage and citrus fruit odour by wild and mass-reared sterile Mediterranean fruit fly males of a genetic sexing strain. *J. Appl. Ent.* 123: 139-143.
- Kouloussis, N.A., B.I. Katsoyannos, N.T. Papadopoulos, C.S. Ioannou and I.V. Iliadis. 2010.** Enhanced mating competitiveness of *Ceratitis capitata* males following exposure to citrus compounds. *J. Appl. Entomol.* DOI: 10.1111/j.1439-0418.2010.01535.x.
- Koyama, J., H. Nakamori and H. Kuba. 1986.** Mating behavior of wild and mass-reared strains of the melon fly, *Dacus cucurbitae* Coquillett (Diptera: Tephritidae), in a field cage. *Appl. Ent. Zool.* 21(2): 203-209.
- Kuba, H. and J. Koyama. 1985.** Mating behavior of wild melon flies, *Dacus cucurbitae* Coquillett (Diptera: Tephritidae) in a field cage: courtship behavior. *Appl. Ent. Zool.* 20(4): 365-372.
- Lance, D.R., D.O. McInnis, P. Rendon and C.G. Jackson. 2000.** Courtship among sterile and wild *Ceratitis capitata* (Diptera: Tephritidae) in field cages in Hawaii and Guatemala. *Ann. Entomol. Soc. Am.* 93(5): 1179-1185.
- Liedo, P., G. Ibarra, A.B. Davila, M.I. Barrios, M.L. Sosa and G. Perez-Lachaud. 1996.** Mating competitiveness of three mass-reared strains of the Mediterranean fruit fly (Diptera: Tephritidae). In IAEA [ed.], Second RCM on "Medfly mating behaviour under field cage conditions", Tapachula, Mexico, 19-23 February 1996. IAEA. Vienna, Austria.
- Liedo, P., E. De Leon, M.I. Barrios, J.F. Valle-Mora and G. Ibarra. 2002.** Effect of age on the mating propensity of the Mediterranean fruit fly (Diptera: Tephritidae). *Fla. Entomol.* 85(1): 94-101.
- Liedo, P., D. Orozco, L. Cruz-López, J.L. Quintero, C. Becerra-Pérez, M. Del Refugio Hernández, A. Oropeza and J. Toledo. 2010.** Effect of post-teneral diets on the performance of sterile *Anastrepha ludens* and *Anastrepha obliqua* fruit flies. *J. Appl. Entomol.*
- Liedo, P., S. Salgado, A. Oropeza and J. Toledo. 2007.** Improving Mating Performance of Mass-reared Sterile Mediterranean Fruit Flies (Diptera: Tephritidae) through Changes in Adult Holding Conditions: Demography and Mating Competitiveness. *Fla. Entomol.* 90(1): 33-40.
- Lux, S.A., F.N. Munyiri, J.C. Vilardi, P. Liedo, A. Economopoulos, O. Hasson, S. Quilici, K. Gaggli, J.P. Cayol and P. Rendon. 2002.** Consistency in courtship pattern among populations of medfly (Diptera: Tephritidae): comparisons among wild strains and strains mass reared for SIT operations. *Fla. Entomol.* 85(1): 113-125.
- Maor, M., B. Kamensky, S. Shloush and B. Yuval. 2004.** Effects of post-teneral diet on foraging success of sterile male Mediterranean fruit flies. *Entomol. Exp. et App.* 110: 225-230.
- McInnis, D.O., D.R. Lance and C.G. Jackson. 1996.** Behavioral resistance to the Sterile Insect Technique by Mediterranean fruit fly (Diptera: Tephritidae) in Hawaii. *Ann. Entomol. Soc. Am.* 89(5): 739-744.
- McInnis, D.O., B.J. Paranhos and T.E. Shelly. 2013.** Survival of sterile male Mediterranean fruit flies in large field cages after release at different ages. *J. Appl. Entomol.* 137 (Supplement 1): S43-S48.
- McInnis, D.O., P. Rendon, E.B. Jang, A. Van Sauers-Muller, R.L. Sugayama and A. Malavasi. 1999.** Interspecific mating of introduced, sterile *Bactrocera dorsalis* with wild *B. carambolae* (Diptera: Tephritidae) in Suriname: a case for cross-species sterile insect technique. *Ann. Entomol. Soc. Am.* 758-765.
- McInnis, D.O., P. Rendon and J. Komatsu. 2002.** Mating and remating of medflies (Diptera: Tephritidae) in Guatemala: individual fly marking in field cages. *Fla. Entomol.* 85(1): 126-137.

- Meats, A. 2007.** Dispersion of Fruit Flies (Diptera: Tephritidae) at High and Low Densities and Consequences of Mismatching Dispersions of Wild and Sterile Flies. *Fla. Entomol.* 90(1): 136-146.
- Meats, A., C.J. Smallridge and B.C. Dominiak. 2006.** Dispersion theory and the sterile insect technique: application to two species of fruit fly. *Entomol. Exp. et Appl.* 119: 247–254.
- Meats, A. and C. J. Smallridge. 2007.** Short- and long-range dispersal of medfly, *Ceratitis capitata* (Dipt., Tephritidae), and its invasive potential. *J. Appl. Entomol.* 131(8): 518–523.
- Morelli, R., B.J. Paranhos, A.M. Coelho, R. Castro, L. Garziera, F. Lopes and J.M.S. Bento. 2010.** Exposure of sterile Mediterranean fruit fly (Diptera: Tephritidae) males to ginger root oil reduces female remating. *J. Appl. Entomol.* DOI: 10.1111/j.1439-0418.2010.01584.x.
- Orozco, D. and R.O. Lopez. 1993.** Mating competitiveness of wild and laboratory mass-reared medflies: effect of male size. 185-188. In Aluja, M., and P. Liedo, [eds.], *Fruit flies: biology and management*. Springer-Verlag, New York, NY, USA.
- Orozco-Dávila, D., R. Hernández, S. Meza and J. Domínguez. 2007.** Sexual competitiveness and compatibility between mass-reared sterile flies and WILD populations of *Anastrepha ludens* (Diptera: Tephritidae) from different regions in Mexico. *Fla. Entomol.* 90(1): 19-26.
- Paranhos, B.J., D. McInnis, R. Morelli, R.M. Castro, L. Garziera, L.G. Paranhos, K. Costa, C. Gava, M. L.Z. Costa and J.M.M. Walder. 2010.** Optimum dose of ginger root oil to treat sterile Mediterranean fruit fly males (Diptera: Tephritidae). *J. Appl. Entomol.* DOI: 10.1111/j.1439-0418.2010.01595.x.
- Pereira, R., N. Silva, C. Quintal, R. Abreu, J. Andrade and L. Dantas. 2007.** Sexual performance of mass reared and wild Mediterranean fruit flies (Diptera: Tephritidae) from various origins of the Madeira islands. *Fla. Entomol.* 90(1): 10-14.
- Pereira, R., N. Silva, C. Quintal, R. Abreu, J. Andrade and L. Dantas. 2007.** Effect of acclimation to outdoor conditions on the sexual performance of mass-produced medflies (Diptera: Tephritidae). *Fla. Entomol.* 90(1): 171-174.
- Pereira, R., J. Sivinski and P. E. A. Teal. 2009.** Influence of methoprene and dietary protein on male *Anastrepha suspensa* (Diptera: Tephritidae) mating aggregations. *J. Insect Physiol.* 55: 328-335.
- Plant, R.E. and R.T. Cunningham. 1991.** Analyses of the dispersal of sterile Mediterranean fruit flies (Diptera: Tephritidae) released from a point source. *Environ. Entomol.* 20: 1493-1503.
- Prokopy, R.J. and J. Hendrichs. 1979.** Mating behavior of *Ceratitis capitata* on a field-caged host tree. *Ann. Entomol. Soc. Am.* 72: 642-648.
- Rendon, P., D.O. McInnis, D.R. Lance and J. Stewart. 2000.** Comparison of medfly male-only and bisexual releases in large scale field trials. 517-525. In Tan, K. H. [ed.], *Area-wide management of fruit flies and other major insect pests*. Penerbit Universiti Sains Malaysia. Penang, Malaysia.
- Robinson, A.S., U. Cirio, G.H.S. Hooper and M. Capparella. 1986.** Field cage studies with a genetic sexing strain in the Mediterranean fruit fly, *Ceratitis capitata*. *Entomol. Exp. Appl.* 41: 231-235.
- Schroeder, W.J. and D.L. Chambers. 1977.** Measuring the startle activity in Mediterranean fruit fly, *Ceratitis capitata*, populations. pp. 35-36 In E. F. Boller and D. L. Chambers [eds.], *Quality Control, An Idea Book for Fruit Fly Workers*. Bulletin 1977/5, WPRS-IOBC.
- Schroeder, W.J., D.L. Chambers, and R.Y. Miyabara. 1973.** Mediterranean fruit fly: propensity to flight of sterilized flies. *J. Econ. Entomol.* 66:1261–1262.
- Segura, D., N. Petit-Marty, R. Sciurano, T. Vera, G. Calcagno, A. Allinghi, P. Gómez Cendra, J. Cladera and J. Vilardi. 2007.** Lekking Behavior of *Anastrepha fraterculus* (Diptera: Tephritidae). *Fla. Entomol.* 90(1): 154-162.

- Shelly, T.E., J. Edu and J. Nishimoto. 2010.** Chilling and flight ability and mating competitiveness of sterile males of the Mediterranean fruit fly. *J. Appl. Entomol.* DOI: 10.1111/j.1439-0418.2010.01532.x.
- Shelly, T.E., S.S. Kennelly and D.O. McInnis. 2002.** Effect of adult diet on signaling activity, mate attraction, and mating success in male Mediterranean fruit flies (Diptera: Tephritidae). *Fla. Entomol.* 85(1): 150-155.
- Sivinski, J., C.O. Calkins and R. Baranowski. 1994.** A container for eclosion and holding adult insects prior to release. *Fla. Entomol.* 77(4): 513-515.
- Sookar, P., I. Haq, A. Jessup, D. McInnis, G. Franz, V. Wornoyaporn and S. Permalloo. 2010.** Mating compatibility among *Bactrocera cucurbitae* (Diptera: Tephritidae) populations from three different origins. *J. Appl. Entomol.* DOI: 10.1111/j.1439-0418.2010.01576.x.
- Stalker, H.D. 1942.** Sexual isolation studies in the species complex *Drosophila virilis*. *Genetics.* 27: 238-267.
- Teal P.E.A., Y. Gomez-Simuta, B.D. Dueben, T.C. Holler and S. Olson. 2007.** Improving the efficacy of the sterile insect technique for fruit flies by incorporation of hormone and dietary supplements into adult holding protocols. 163-174. In Vreysen, M. J. B., A. S. Robinson and J. Hendrichs. *Area-wide control of insect pests. From research to field implementation.* Springer, Dordrecht, The Netherlands.
- Thomas, D.B. and J. Loera-Gallardo. 1998.** Dispersal and longevity of mass-released, sterilized Mexican fruit flies (Diptera: Tephritidae). *Environ. Entomol.* 27(4): 1045-1052.
- Vera, M.T., R.J. Wood, J.L. Cladera and A.S. Gilburn. 2002.** Factors affecting female remating frequency in the Mediterranean fruit fly (Diptera: Tephritidae). *Fla. Entomol.* 85(1): 156-164.
- Vreysen, M.J.B. 2005.** Monitoring sterile and wild insects in area-wide integrated pest management programmes. 325-362. In Dyck, V. A., J. Hendrichs and A. S. Robinson. [eds.], *Sterile Insect Technique. Principles and Practice in Area-Wide Integrated Pest Management.* Springer, Dordrecht, The Netherlands.
- Vreysen, M.J.B., K.M. Saleh, R. Lancelot and J. Bouyer. 2011.** Factory tsetse flies must behave like wild flies: a prerequisite for the sterile insect technique. *PLoS Negl Trop Dis* 5(2): e907. doi:10.1371/journal.pntd.0000907
- Weldon, C. and A. Meats. 2007.** Short-range dispersal of recently emerged males and females of *Bactrocera tryoni* (Froggatt) (Diptera:Tephritidae) monitored by sticky sphere traps baited with protein and Lynfield traps baited with cue-lure. *Aust. J. Entomol.* 46: 160–166.
- Wong, T.T.Y., J.I. Nishimoto and H. Melvin Couey. 1983.** Mediterranean fruit fly (Diptera: Tephritidae): further studies on selective mating response of wild and of unirradiated and irradiated, laboratory-reared flies in field cages. *Ann. Entomol. Soc. Am.* 76(1): 51-55.
- Yamamura, K., M. Kishita, N. Arakaki, F. Kawamura and Y. Sadoyama. 2003.** Estimation of dispersal distance by mark-recapture experiments using traps: correction of bias caused by the artificial removal by traps. *Popul. Ecol.* 45:149–155. DOI 10.1007/s10144-003-0152-x.
- Yuval, B., M. Maor, K. Levy, R. Kaspi, P. Taylor and T. Shelly. 2007.** Breakfast of Champions or Kiss of Death? Survival and Sexual Performance of Protein-fed, Sterile Mediterranean Fruit Flies (Diptera: Tephritidae). *Fla. Entomol.* 90(1): 115-122.
- Zapfen, G.I., J. Hendrichs, P. Liedo and A. Cisneros. 1983.** Comparative mating behaviour of wild and mass-reared sterile medfly *Ceratitis capitata* (Wied.) on a field cage host tree - II. Female mate choice. 397-409. In Cavalloro, R. [ed.], *Fruit flies of economic importance.* A.A. Balkema. Rotterdam, the Netherlands.

8. Ancillary tests

8.1. Activity Test

Objective

To confirm that sterile males are less reactive than wild flies, probably as an effect of selective pressures under the mass-rearing environment.

Discussion

As stated in the Fly Irritability Tests (section 7.4), laboratory flies can present a decreased pattern of activity, fly less and respond slower to adverse stimuli, which results in a major sensibility to predators. This can be quantified and the change in behavioural patterns determined with detailed 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 a 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 being sexually mature and once they are already mature.

Equipment

- All the required equipment to obtain adult flies.
- Recording cages: Transparent Plexiglas cages of approximately 1-2 L to place flies.
- Digital video camera.
- Computer with specific software to analyse recordings.
- Artificial lighting.

Procedures

Procedures can vary whether flies are to be tested before or after sexual maturation and whether it is desirable to see differences in activity as a result of the presence of individuals of the opposite sex. At the appropriate age to test the flies, place 3-5 flies of one sex (in case 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. Classify the day in different moments (early morning, midday and afternoon and dusk) and record for each moment several groups (at least 5).

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). The presence of differences in the activity pattern

between wild and mass-reared flies may indicate that the mass-rearing environment is selecting for less active flies either because highly active flies are more prone to face physical damage (collisions) than less active flies, spend much energy in fights or agonistic interactions that reduce their survival. In addition, under artificial 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.

8.2. 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

Lekking species show elaborate courtships 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 by releasing a lower number of flies, preferably individually labelled, and observing the behaviour of them during the entire period of the test. The procedures to extend these observations are in general open and will depend on the particular characteristics of the species in point. Here we provide some guidance.

Source and Handling of Flies

Wild flies and sterile laboratory males: As for the mating performance field cage test. However, in this case, it is recommended to label the flies individually in order to be able to identify each fly and record their 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) in the thorax (**Figure 8.1**). Marking the females is up to the particular objective of this test.



Figure 8.1: 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 including standard round outdoor field cages with host trees.
- Material to label individually the flies: water based 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 8.2**) 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 inflate pouches on the pleura. Calling males may intermittently vibrate or "fan" their wings while standing in place; during fanning, anal sac is partially retracted and held under the abdomen, and thus it is more difficult to observe.



Figure 8.2: *Ceratitidis 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.

The observer can assess for each individual 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 among 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 and possibly the pheromone emitted is different, and/or (2) females that were attracted to sterile males seldom copulated with them.

Periodicity of calling can be evaluated by computing median or mean time at which sterile and wild males were observed calling in each cage and then analysing the resulting values with a paired t-test. If it is proven that sterile males are actively calling but even though they are rejected by the approaching females, then it is desirable to complete this observations with the "Pheromone Compatibility Test", described in section 8.5.

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 the occurrence of mixed leks (the two populations/strains merge in the same leks) or spatial isolation. Moreover, this detailed observational studies permits 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 contribute to decide whether rearing conditions can be adjusted or the strain replaced in order to restore competitiveness.

8.3. Pheromone Compatibility 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. There is the possibility that pheromone production may be reduced under this circumstance. There is also the possibility that the ratio of the components of the pheromone may vary or change. If any of these situations occur, this could affect how the wild target female would react to the sterile male in the mating arena. The following describes a simple test which can be included while the Mating Performance Field Cage Test (Section 7.2) is being conducted.

Source and Handling of Flies

Wild flies and sterile laboratory males:

As for the Mating Performance Field Cage test (section 7.2); however in this case it is extremely important that wild flies are matured in a distinct room than laboratory flies to avoid any contact of volatiles between individuals of different origins. If possible wild females should be in a separate room also.

Equipment

- All the required equipment for the mating performance field cage test (Section 7.2) including standard round outdoor field cages with host trees.
- Nine small-screened cages (10 x 5 x 5 cm) with 2/3 m long cord and hooks on ends to suspend cages inside each of the standard field cages.
- Aspirator.
- Clipboard and forms.

Procedures

Place 5 sterile males in each of 3 small cages, 5 wild males in each of 3 small cages and keep 3 small cages empty. Suspend the small cages from branches in the shade of the tree(s) at an equal distance from each other. Avoid two cages containing the same type of male to be adjacent to each other. Release 100 wild females inside the field cage. After 10 minutes, count the number of female flies attached to the screens of each cage and record the number on the form. Then gently blow the female flies from the small cages and rotate the small cages. Repeat the test every 10 minutes for 9 periods so that each small cage has occupied each position in the tree cage. Total the number of females for each treatment (male type) and determine the mean and the standard error.

Interpretation

Based on experience, one would expect the sterile males 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. The empty cages should have no or very few females resting on them (the presence of high number of females there, is an indicator that females are being attracted to the cage for some other reason and the cage should be changed). If there are substantially more females resting on the wild male cages than on the sterile male cages, there may be some concern that the pheromone has either changed or the sterile males are not producing enough pheromone. This could conceivably affect the compatibility of the sterile male and the wild female in the field.

8.4. Male Frequency of Mating Test

Objective

Determine the number of matings a sterile male can accomplish with successful sperm transfer and/or reduction of female receptivity.

Discussion

The sterile insect technique relies in the ability of sterile males to transfer mobile (yet non-fertile sperm) in the female so that the female uses this sperm to inseminate their eggs. Other substances transferred by the male in the ejaculate originated in the accessory glands and composed mainly by proteins, alter female behaviour reducing their receptivity to further matings. For some species it seems that the males are able to regulate the amount of sperm transferred and they don't deplete their sperm loads in one copulation.

In sterile insects the spermatogenesis is interrupted and hence the amount of sperm available at the time of release is all they have to transfer to the females throughout their lives. Assessing how many copulations are required to deplete this load and how many days this may take can give a more detailed information on how many matings each sterile male can achieve and may help to estimate the sterile:wild effective ratio. In the absence of such information, it should be assumed that each male copulates only once. This evaluation can be performed in conjunction with the previous observation on detailed aspects of male sexual behaviour and taking advantage of individual labelling of the males. In addition, it can be run in field cages for the first day and transferred to the laboratory for consecutive days if weather conditions are not constant along successive days and may alter flies' behaviour. An additional morphometric analysis of the males may contribute to determine the effect of male size on sperm transfer.

Source and Handling of Flies

Wild flies and sterile laboratory males:

As for the mating performance field cage test, but in this case males should be labelled individually as in the Lekking and Sexual Behaviour observations. It is not necessary to mark wild females and additional wild females will be required to be released after the couple disengage. Extra males will be required if female receptivity on subsequent days is evaluated. If wild males are scarce, it is better to use laboratory fertile males to ensure all males are uniform and of the same age and nutritional status.

Equipment

- All the necessary equipment as for the Mating Performance Field Cage Test.
- Laboratory mating cages (i.e. of adequate size with a branch or small plant inside).
- Dissecting microscope.
- Dissecting forceps.
- Glass slides and cover-slides to place dissected spermathecae.
- Microscope to determine sperm transfer.

Procedures

On the day of the test, wild and sterile males are released into the field cage and given a period of time (e.g., 15-30 min) to disperse and establish territories. The number of flies to be released will depend on the labelling technique (probably no more than ten with colour marking and up to 100 with the small letters printed in papers of different colours). Time of release should precede the time of peak mating activity and after the period of acclimatization of the males, the chosen number of wild females are released. Seek for mating couples, collect them and place them in the shadow to allow copulation to take place with no stress. Record in the form the time the copulation started and the identity of the male. Once the pair finishes copulation, return the male to the field cage and replace the female for a virgin one. This procedure should be done carefully and with the aid of a manual aspirator. Keep the mated female to score later in the lab the presence of sperm in their spermathecae. Even if the mated female flies away, release a new virgin one.

Take the mated females to the laboratory to either determine sperm transfer or reduction in the receptivity (details on both procedures are provided in the Female Propensity to Remate Test, Section 8.6 below). Continue the observation until well passed the time of sexual activity of the species. Recover all the males from the cage and maintain them until the next day when the procedure is repeated. The test should last until no sterile males mate or no sperm transfer is observed. To avoid impact of weather conditions, especially rain, this test can be done in a greenhouse.

Note on sperm counts: amount of sperm stored in the spermathecae has been proven to be related to female renewal of receptivity. The counting technique in itself is not difficult but it is very time consuming and hence, can be left for research purposes.

Interpretation

Compute the number of matings each male achieved and compare according to male origin (wild vs. sterile). Determine for each case the length of the copulation and the interval between successive copulations (i.e. refractory period). Compare means with a t-test. Determine the number of mating that resulted in sperm transfer and correlate this with the male origin, status (virgin vs. mated) and previous refractory period in the case of already mated males. All these data will provide detailed information on how many successful matings each sterile male is able to achieve and how long it takes to recover from one mating in order to engage in another mating. In addition, it will provide an idea of how many of those males that did not mated in the first day, mate in the second or successive days, reflecting a more accurate proportion of the released males that engage in mating. This information will provide an effective number of the sterile matings being released per released male. If the variable recorded is the renewal of female receptivity instead of the presence of sperm, the analysis and interpretation is the same.

8.5. Female Propensity to Remate Test

Objective

To compare the renewal of female receptivity between females mated 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 is 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 renewal of female receptivity after on subsequent days the first mating. If wild males are scarce, laboratory fertile males can be used at this point. This will ensure all males are uniform and of the same age and nutritional status.

Equipment

- All the necessary equipment as for the Mating Performance Field Cage Test (Section 7.2).
- Laboratory mating cages (i.e. of adequate size with a branch or small plant inside).
- Dissecting microscope.
- Dissecting forceps.
- Glass slides and cover-slides to place dissected spermathecae.
- Microscope to determine sperm transfer.

Procedures

The test is a continuation of the Mating Performance Field Cage Test (section 7.2). In the first day it is extended until no sexual activity is recorded or preferably during all day in those species that mate at any time in the day. Each female is identified after the first mating and on subsequent days, they are offered new virgin males and the rate of remating is recorded. It is recommended then that this part is held under laboratory conditions to avoid dependency on weather conditions. The concept in itself can be generalized but a detailed protocol should be adapted to each species in particular. 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 l) or with other females that have mated with males of the same type (12 - 20 L). Special care should be taken not to mixed females that mated with males of different type. On the next day or after a

certain interval according to the mean time of the known refractory period for this species, the female is offered two sexually matured yet virgin males during the time of sexual activity to determine renewal of 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. The test should be run at least twice the time of the mean refractory period (if known for the given fruit fly species) and virgin males should be offered at regular intervals (i.e. every day or every two days). If possible, an oviposition substrate can be provided to the females and the number of eggs laid recorded to be used as a covariable in the analysis. If female receptivity is very sensitive to fly density, then it is recommended to run the test under relaxed conditions in order to allow the females reject the males.

If desired, a set of females of each type can be used to determine sperm transfer and amount 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 soap. With the aid of entomological pins the volume is stirred to allow sperm to disperse. Then, the slide is covered with a coverslip and the number of sperms present in a certain number of fields under a microscope is recorded. The amount of fields will depend on the magnification used and it is recommended to consider around 10% of the total area of the coverslip (for further details see Taylor *et al.* 2000; Perez-Staples and Aluja 2006; Abraham *et al.* 2011 and references therein).

Interpretation

For meaningful data, 25 females of each category should be analyzed. Remating rate among 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 (nutritional status, size, exposure to male enhancers, 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 laboratory 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 modulating 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.

8.6. The Fried Test

Objective

To provide an estimate of the overall mating competitiveness of sterile male's 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 in itself 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 7.2) whenever possible.

Source and Handling of Flies

a) Wild flies and sterile laboratory males:

As for the mating performance field cage test (section 7.2), but it is not necessary to mark (paint) the flies if the test is to be run separately from this test.

b) Fertile laboratory 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 transferred 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:

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

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.

8.7. Open 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 Fried test described above provides the closest measurement 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. 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. For this reason this test is appropriate in areas where sterile releases have just started or where the wild population is still high.

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 under a routinely scheme and a close 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. Revise 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 not be as good as 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 above. Use the data from the 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 normal 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, being not possible to assure that matings are not taking place there. This can be minimized by placing the traps in the field in those times of the day where 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. 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 and a wild male or only with one of them 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 molecular markers have been reported. In addition, it is also labour consuming and requires sending the material to laboratories that can perform the analysis.

8.8. Survival in Field Cages

Objective

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

Discussion

Sterile insects are released in the field in which they must survive and mate to achieve population control. 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 it is designed to document sterile males' survival under field conditions in order to plan and/or modify production and handling procedures, as well as periodicity of the release activities.

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. From underneath the cage a zipper closed opening (**Figure 8.3**) allows to introduce a potted plant of the desired species (e.g. coffee potted plant). Whenever possible, it is recommended that these plants are the same plant species under which sterile males will be released. Potted plants should be placed in the field under the same conditions

of the release area. This would allow them to collect bird droppings, dew and other elements that sterile males will normally find in the field. Prior to the test, potted plants are introduced to the cages. Inside the cages, branches of potted plants serve as feeding, resting surface and mating arenas. Small field cages can be placed at locations close to the release area, to include variables such as elevation and all local climatic conditions. Under all these conditions this test should be very close to the actual survival of the insects in the field, disregarding in this case insect predation; evaluation that deserves a test set up in itself, as insect interactions with predators will vary according to the specific conditions of each area.



Figure 8.3: 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 to release in the field cage flies that were recovered from the releasing machine as in the Absolute Fliers Post Release Test (see section 6.4.1). If wild flies are to be released in the cages as well, then procedures should resemble that of the Mating Performance Field Cage Test (section 7.2).

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 where the sterile males are released.
- Recording forms.

Procedures

Place the cages at the desired field location (**Figure 8.4**) and recover sterile males from the releasing machine as proposed in the Absolute Fliers Post Release Test. Release the flies inside the cages (100 insects/cage is recommended). If sterile and wild flies are released

inside each cage, then flies should be labelled. After 48-72 h (depending on the species) open the lateral zippers and register the number of dead flies. Record the number in the form. During the test it is important to provide within the cage the microhabitats the fly will encounter in the area of release in order to resemble natural conditions. To keep track of this, it is advisable to record weather conditions at regular intervals (once an hour) for which it is appropriate to include data loggers within the cages.



Figure 8.4: 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 alive flies 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. It is also possible to adjust the frequency at which the cages will be revised and to do it more than once during the test (i.e. very 12-24 h) and for a longer period (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).

8.9. Pupal Size

Objective

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

Discussion

Size is a valuable indicator of overall viability of pupae and correlates with size of the resulting adult flies. Evidence shows that larger 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 size will vary depending upon the strain and rearing system, so using size 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.

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 8.5**). 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 size 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 8.5: 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 size 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:

<i>C. capitata</i>	1.4 mm to 1.9 mm
<i>A. suspensa</i>	1.8 mm to 2.5 mm
<i>A. obliqua</i>	1.3 mm to 2.9 mm
<i>A. ludens</i>	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 size of pupae produced by a facility can result from poor nutrition, overcrowding in the larval stage, high temperatures in the larval diet, inappropriate holding conditions during pupal maturation, or other factors that could reduce the viability of the released insects. As small size 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.

8.10. Relevant literature

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

Abraham, S., L. Goane, J.L. Cladera y 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. Talyor. 2010. Activity pattern of Queensland fruit fly (*Bactrocera tryoni*) are affected by both mass-rearing and sterilization. *Physiol. Entomol.* 35: 148-153

DRAFT

9. Forms for Recording Quality Control Data

9.1. Pupal Weight Assessment Form

Set-up Date: _____

	Shipment Date	Lot #	Collection Date	Test Date	Tester
<input type="checkbox"/> Pre Irradiation					
<input type="checkbox"/> On Arrival					

		Replicate					
		1	2	3	4	5	Sum
	Total Pupae						
	Total Weight						

Calculations:

		Value
	$\left\{ \frac{\sum_{i=1}^5 \text{Replicates}}{\sum_{i=1}^5 \text{Total Pupae}} \right\}$	Average Weight (5 samples)

Observations:

Authorization:

9.2. Emergence and Flight Ability Assessment Form

Set-up Date: _____

☐ Pre Irradiation

☐ Post Irradiation

☐ On Arrival

Shipment Date	Lot #	Collection Date	Test Date	Tester

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

Calculations:

			Value
E	$T-(A+B)/100$ Assumes 100 pupae per replicate	% Emergence	
F	$T-(A+B)/100$ Assumes 100 pupae per replicate	% Fliers	
	F/E	Rate of Fliers	

Observations:

Authorization:

9.3. Survival under Stress Assessment Form

Set-up Date: _____

- ☐ Pre Irradiation
☐ Post Irradiation

- ☐ At arrival
☐ Post Handling/
Chilling
☐ Post Release
☐ Post Handling/
Chilling (w/water)
☐ Post Release
(w/water)

Shipment Date	Lot #	Collection Date	Test Date	Test Time	Tester

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

Calculations:

			Value
	DM/100 Assumes 100 pupae per replicate	% Male Survival	
	DF/100 Assumes 100 pupae per replicate	% Female Survival	

Observations: _____

Authorization: _____

9.4. Sex Ratio and Timing of Emergence Assessment Form

Set-up Date: _____

☐ Post Irradiation

☐ On Arrival

Shipment Date	Lot #	Collection Date	Test Date	Tester

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

Calculations:

		Value
M/(M+F)x100 Assumes 100 pupae per replicate	% Male	

Observations:

Authorization: _____

Set-up Date: _____

☐ Post Irradiation

☐ On Arrival

Shipment Date	Lot #	Collection Date	Test Date	Tester

	Replicate											
Hours post hypoxia	1		2		3		4		5		Sum	
	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: _____

9.5. Datasheet for Shipment of Sterile Pupae

A copy of this datasheet should be present within each box of the consignment.

Name and address of the facility (origin):	Name and address of the recipient:
.....
.....
.....
.....

Consignment General Information	
Irradiation date:	Irradiation dose (Gy):
Packing date:	Shipping date:
Total No of boxes:	Total weight (kg):

Elements	Box Number within the Consignment										Total	Observations
	1	2	3	4	5	6	7	8	9	10		
Number of pupae containers inside the box											<i>a</i>	
Weight (kg)											<i>b</i>	
Number of pupae containers with radiation sensitive indicator											<i>c</i>	
Number of indicators that were exposed to the recommended dose											<i>d</i>	
Number of indicators countersigned at the origin, after irradiation											<i>e</i>	

Observations: _____

Authorization: _____

9.6. Sterility Assessment Form

Set-up Date: _____

☐ Post Irradiation

☐ On Arrival

Shipment Date	Lot #	Collection Date	Test Date	Tester

		Replicate		
Egg Collection Day		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/Hx100	% Sterility		

Observations:

Authorization:

9.7. 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				

¹State the exact number of flies effectively released in the cage considering replacements. ²Here 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).

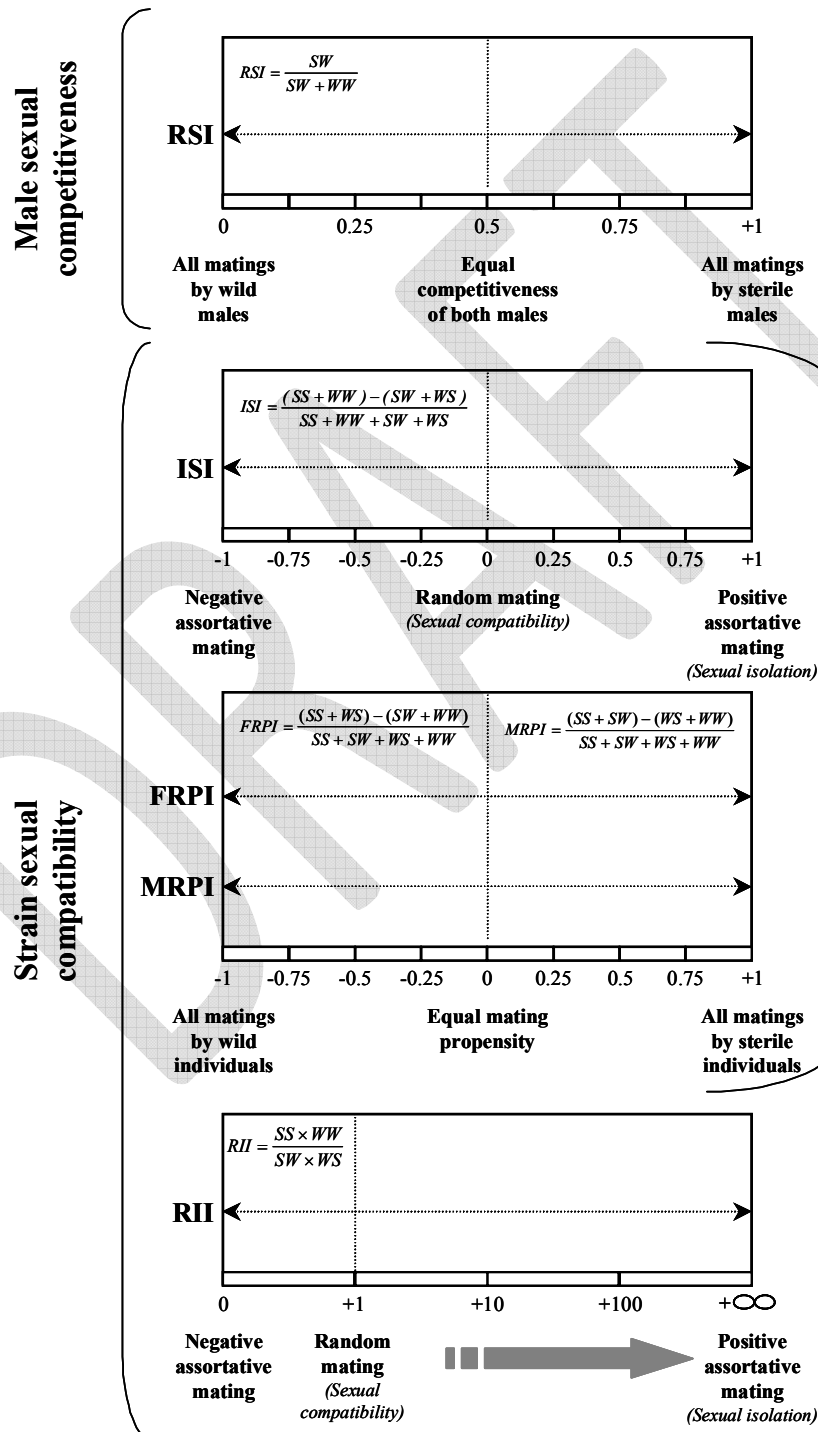
Σss	Σsw	Σws	Σww

Observations: _____

9.8. 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 \pm standard error).



9.9. 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				

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

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

³Here 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 Wädenswil, 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. 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. Final proceedings will appear 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. 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.

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

DRAFT

Appendix B: Known Sources of Key Equipment and Supplies⁹

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.	

⁹ 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)]	Agrisense - BCS Ltd. Treforest Industrial Estate, Pontypridd Mid Glamorgan CF37 5SU, United Kingdom. Tel.: (+44) 1443841155, Fax: (+44) 1443844205 American Scientific & Industrial Supplies PO Box 8247, Radnor PA 19087, USA. Tel.: (+1) 6109647665, Fax: (+1) 6109641860 E-mail: sales@asi-supplies.com
Attractant [torula yeast pellets]	Scentry Biologicals Inc. (<i>see above</i>)
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
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	<p>Better World Manufacturing Inc. (see above)</p> <p>Gempler's Pest Management Supply P.O. Box 270, 211 Blue Mounds Rd., Mt. Horeb, WI 53572, USA. Tel.: (+1) 8002727672, Fax: (+1) 8005511128</p> <p>Scentry Biologicals Inc. (see above)</p> <p>Sorygar S.L. (for Tephri® traps) Quinta del Sol n. 37, Las Rozas - Madrid 28230, Spain. Fax: (+34) 916407000 E-mail: sorygar@nexo.es</p> <p>Suterra (formerly Consep, Inc.) (see above)</p>
As described in 0 3.3. IRRADIATION AND PROCESS Control	
Dose-specific radiation-sensitive indicators [70, 125, 300 Gy]	<p>International Speciality Products (ISP) Technologies Inc. 1361 Alps Rd, Wayne, New Jersey, 07470, USA. Tel.: (+1) 2016284000, Fax: (+1) 2016283016</p>
As described in 0 4.1. DOSIMETRY System	
Gafchromic® dosimetry media [type HD-810, package of 5 sheets, 8" x 10 "]	<p>Elimpex Medizintechnik GesmbH <u>Spechtgasse 32, A-2340 Moedling, Austria.</u> <u>Tel.: (+43) 2236410450, Fax: (+43) 2236410459</u> <u>E-mail: falk@elimpex.com</u> <u>(specify "catalog number 37-040")</u></p> <p>ISP Technologies Inc. (see above)</p>
Digital Radiachromic® Reader System [model FWT-92D-220]	<p>Far West Technology Inc. 330 D South Kellogg, Goleta CA 93117, USA. Tel.: (+1) 8059643615, Fax: (+1) 8059643162</p>
Miscellaneous	
Fluorescent microscope [trinocular with phase contrast, model VanGuard 1486FL]	<p>Service for Science and Industry (SFSI) Inc. <u>1101 North Kings Highway, Suite 201, Cherry Hill NJ 08034, USA.</u> <u>Tel.: (+1) 8563210635, Fax: (+1) 8563210636</u> <u>E-mail: sfsi@sfsi-usa.com</u></p>
Multi-use temperature and/or humidity monitors	<p>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</p>

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>).

DRAFT

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 risk¹ from transboundary² 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.

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.

EXECUTIVE SUMMARY

- 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, *Ceratitidis 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 analysis³. **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).

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).

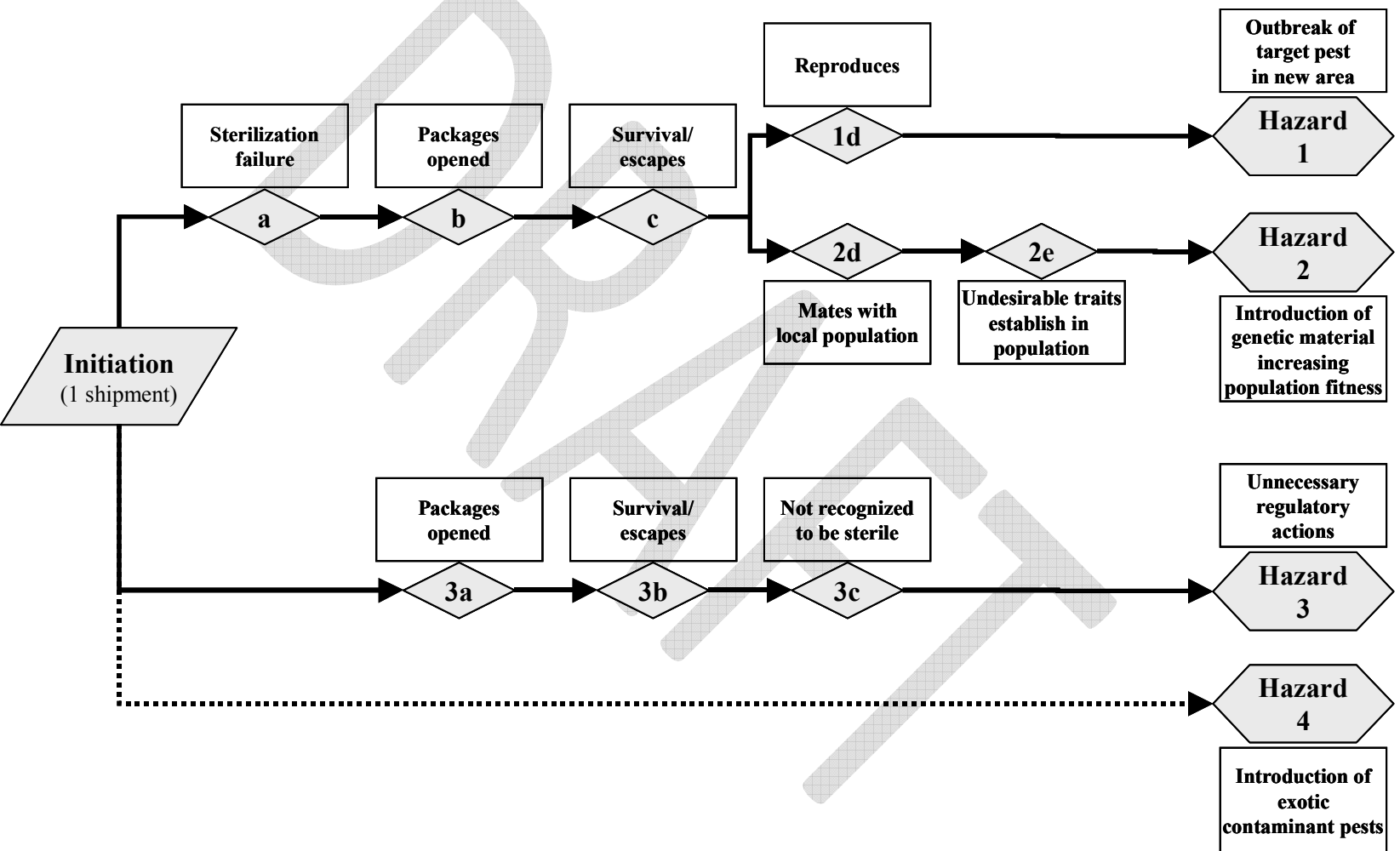


Figure 1. Hazard Scenarios for Transboundary Shipment of Sterile Insects.

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 1×10^{-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 1×10^{-3}*

Event d: Reproduces

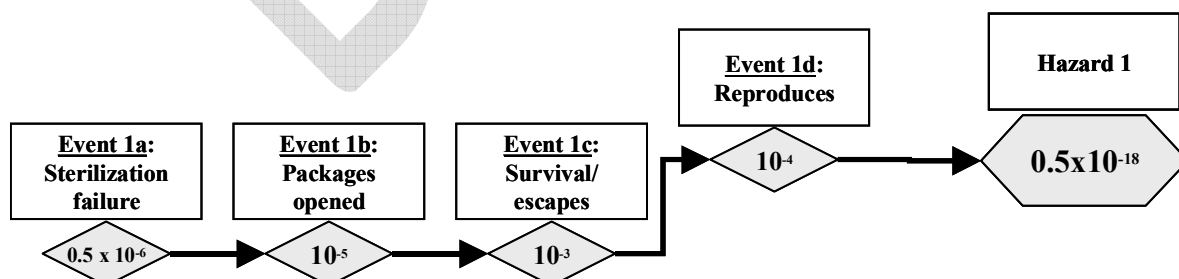
In addition to the above events, reproduction by the escaped insects would be unlikely because:

- 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 1×10^{-4}*

For the scenario for hazard 1 the likelihood of all four events occurring was estimated as a negligible risk with a probability of 0.5×10^{-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

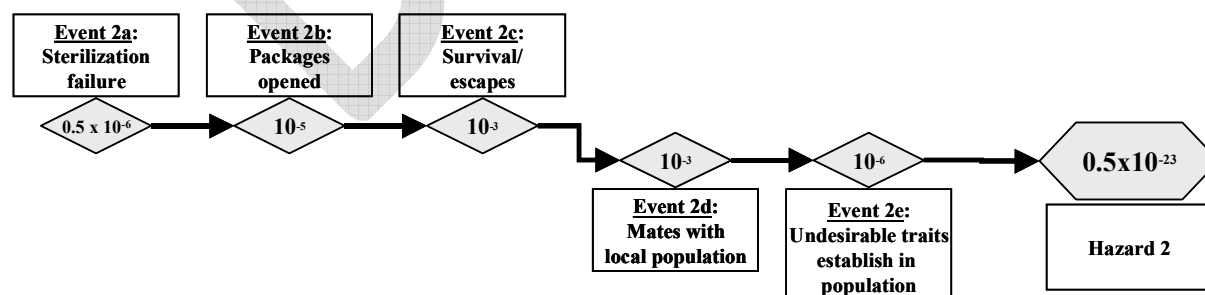
In addition to the above events, the escaped insects would have to possess traits that convey a selective advantage leading to increased fitness. Furthermore, these traits would have to become established in the population. However, this is extremely unlikely because:

- 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 lose 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.

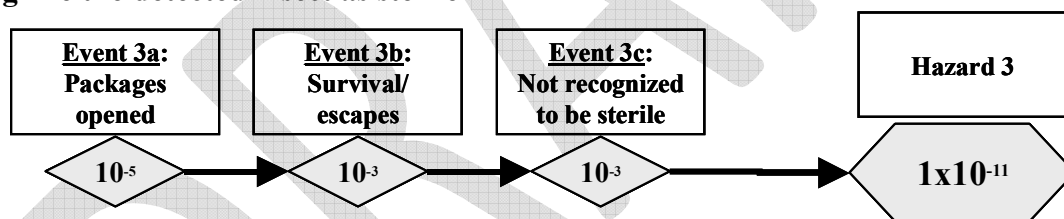
For this to occur the insect must be of regulatory significance:

- 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



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 manuals¹ (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 procedures⁴ 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.

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.

FAO/IAEA (2000) Gafchromic® Dosimetry System for SIT, Standard Operating Procedure. Joint FAO/IAEA, Division of Nuclear Techniques in Food and Agriculture. Vienna, Austria, 42 pp.

FAO, IAEA and United States Department of Agriculture (USDA) (1998) Product Quality Control, Irradiation and Shipping Procedures for Mass-Reared Tephritid Fruit Flies for Sterile Insect Release Programs. Recommendations reached by consensus by an international group of fruit fly quality control experts. 52 pp.

Other references

Food and Agriculture Organization of the United Nations (FAO) (1992) The new world screwworm eradication programme-North Africa 1988-1992. 192 pp.

International Plant Protection Convention (IPPC) (1996) Code of conduct for the import and release of exotic biological control agents. ISPM Pub. No. 3. FAO, Rome.

IPPC (1997) New revised text approved by the FAO conference at its 29th Session- November 1997. FAO, Rome.

IPPC (1998a) Guidelines for surveillance. ISPM Pub. No. 6, FAO, Rome.

IPPC (1998b) Determination of pest status in an area. ISPM Pub. No. 8, FAO, Rome.

IPPC (1998c) Guidelines for pest eradication programs. ISPM Pub. No. 9, FAO, Rome.

IPPC (2001a) Glossary of Phytosanitary Terms. ISPM Pub. No.5, FAO, Rome.

IPPC (2001b) Pest risk analysis for quarantine pests. ISPM Pub. No. 11, FAO, Rome.

Miller L., M. D. McElvaine, R. M. McDowewell and A. S. Ahl (1993) Developing a quantitative risk assessment process, Rev. sci. tech. int. epiz.,1993,V12(4), 1153-1164

Nagel, P. (1995) Environmental monitoring handbook for tsetse control operations. The scientific environmental monitoring group (SEMG) (ed.) Weikersheim: Markgraf 323 pp.

Orr, Richard L., Susan D. Cohen and Robert L. Griffin (1993) Generic Non-indigenous pest risk assessment process, “the generic process” (for estimating pest risk associated with the introduction of non-indigenous organisms). USDA/APHIS Policy and Program Development publication. 40 pages.

Rohwer, G. Gregor (1987) An Analysis of the Mediterranean Fruit Fly Eradication Program in California, 1980-82. USDA/APHIS/PPQ publication. 20 pages.

United States Department of Agriculture (USDA)/Animal and Plant Health Inspection Service (APHIS) (1991) Guatemala MOSCAMED Program. Environmental Analysis. 71 pages.

USDA/APHIS (1992) Risk Assessment: Mediterranean fruit fly. 113 pages.

USDA/APHIS (1993) The economic impact assessment of the Mediterranean fruit fly cooperative eradication program. 27 pages.

USDA/APHIS (1993) Medfly Cooperative Eradication Program. Final Environmental Impact Statement. 184 pages.

USDA/APHIS (1998) Medfly Cooperative Eradication Program, Central Florida, Environmental Assessment. 6 pages.

USDA/APHIS (1998) Medfly Cooperative Eradication Program, Southern Florida, Environmental Assessment. 12 pages.

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.

DRAFT

Appendix E: History of Transboundary Shipments of Sterile Tephritid Fruit Flies (1963-2013)

Year	Tephritid species	Site of production	Amount shipped (million pupae)	Recipient	Observations
1963-1990	Mexican fruit fly, <i>Anastrepha ludens</i>	Monterrey, Mexico	Unknown	Texas, USA	
1970/71	Mediterranean fruit fly, <i>Ceratitis capitata</i>	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, <i>A. obliqua</i>	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 suppress/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	Mediterranean fruit 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 and 2012	Mediterranean fruit fly	Bio-Fly, Israel	369	Croatia	In support of pilot suppression programme
2011 and 2013	Mediterranean fruit fly	Valencia, Spain	425	Croatia	In support of pilot suppression programme

DRAFT