REQUIRED PERIODIC QUALITY CONTROL TESTS

3. Required Periodic Quality Control Tests

3.1. Mating Performance Field Cage Test

Overall Objective

The overall objective of mating performance field-caged test is to release in a confined semi-natural environment (i.e. field caged host tree) sexually mature yet virgin flies from a wild population (ideally from the "target" population of the SIT program or from geographically closest population) and from the laboratory strain that will be used in the operational program, in order to observe their mating behaviour and interactions during the time of sexual activity. The aim is to determine whether the "sexual behaviour" of the flies from the laboratory strain is similar with that of the target wild population.

Specific Objectives

a) ***Assessment of "strain sexual compatibility":***

To determine, by analysing the number of mating couples obtained in each mating combination (four when both males and females of each population/strain are released), the degree of compatibility or sexual isolation between the two populations/strains.

b) ***Assessment of "male sexual competitiveness":***

To determine the ability of sterile male flies to compete with wild males for mating with wild females under semi-controlled field conditions. This is done by analysing the respective number of mating with wild females achieved by the wild and the laboratory males.

c) ***Additional procedures:***

Additional procedures may be included to the above-mentioned test to assess the following: (1) incidence and timing of "calling" (release of pheromone) by sterile and wild males, (2) duration of mating, (3) periodicity of mating, (4) frequency of remating, and (5) overall competitiveness of flies based on resulting hatch of eggs (known as the Fried test).

Discussion

Mating performance field cage test with host trees is the best compromise between laboratory conditions and costly and impractical field observations to assess tephritid fly mating behaviour under semi-natural conditions.

Sexual compatibility is the degree to which two sympatric groups of animals 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 operations. 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 be used for field operations or whenever a significant decrease of male sexual competitiveness (see below) is detected that may lead to what has been referred to as "behavioural resistance".

Male sexual competitiveness refers primarily to how readily wild females accept sterile males (as opposed to wild males) as mates. It was shown that effects of a laboratory rearing system could bring about changes in a rearing colony that can reduce the acceptability of laboratory- or factory-reared males to wild females most notably in shortening some sequences of the male courtship or in changing qualitatively the pattern of some sexual behaviours. 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. Any significant decrease in the competitiveness should lead to the immediate replacement of the strain under mass-rearing before it affects the overall effectiveness of the SIT programme.

Other versions of field-cage test are possible, however the standard design specified here is based on the conclusions of a group of experts who jointly participated in a FAO/IAEA Coordinated Research Project (CRP) on medfly mating behaviour for 5 years. Data from these tests can be used to generate 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. However, it was concluded during this CRP that the nutritional

status, sex ratio and density of flies in relation to available canopy surface in the field cage influences test results. Efforts should thus be made to strictly follow the standards described thereafter.

Figure 9: Standard walk-in field cage used for mating compatibility test

Sources and Handling of Flies

a) ***Sterile flies:***

Place several thousand irradiated (see chapter 4 IRRADIATION PROCEDURES), dyed (1.5g Day-Glo powder per litre) pupae in devices used for "Emergence and Flight Ability Tests" (see 2.2 PERCENT EMERGENCE AND FLIGHT ABILITY), within a screen or Plexiglas cage. Within a few hours of adult emergence, select only flying adults and separate the sexes. Sexing has to be 100% effective; female cages in which even one male is detected cannot be used for the tests and will have to be discarded. Then, hold the flies according to the day of emergence in laboratory cages (screen or Plexiglas) containing water and a protein:sucrose (1:3) diet until sexually mature. Age of sexual maturity will vary with species and strain. Preferably, the sexes should be segregated into two separate rooms at a maximum density of ±40 flies per litre volume. Cages may be held indoors at ≈25°C with a ≈14:10 L:D cycle, although best results may be obtained by holding flies outdoors in a shaded, protected location (e.g., an insectarium) in the vicinity where tests will be run. In any case, moderate temperatures and humidity, clean water (cotton wick changed regularly) and daily light rotation of cages should be maintained to promote survival and to limit unnecessary stress.

b) ***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, however, whenever possible, medium-sized fruits such as guavas, oranges, peaches etc should be preferred, whenever and wherever possible to small fruits (such as cherries, coffee berries etc) which tend to dry rapidly and produce unnaturally smaller flies. Infested fruits of the selected host/variety are then collected directly from the untreated host plant and returned to the laboratory. They are then placed on wide mesh trays or racks over a pupation substrate such as lightly moistened sand or vermiculite. Fruit-holding methods vary widely with the fly species, fruit, and local conditions. The substrate is then gently sifted every 2 days and wild pupae are collected. Pupae are then placed in Flight Ability devices and treated exactly as sterile flies. Only good flyers should be selected and sexed, and adult flies should be maintained in similar conditions to those of sterile flies.

Age of sexual maturity may vary with species and geographic strain; preliminary tests may be required to determine the appropriate age for mating performance field cage test. Flies should, of course, be sexually mature, but flies that are presumably well past their "normal" age of mating in the field should not be used because unmated wild females, as they age, may become more and more willing to accept less-than-optimal males as mates.

Equipment

- Outdoor field cage, ≈2.3 m tall by ≈3 m in diameter, set up over a plant that fills a large portion of the volume of the cage (**Figure 9**). The plant should be a local host plant of the fly species to be tested. Ideally, the plant should be rooted in the ground, but potted plants may suffice if ground-rooted plants are unavailable. Care must be taken in setting up the cages. 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 (citrus, guavas, etc.) should be available per fly released in the field cage. If adequate foliage and light are available within the cages and if not 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. 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 any case, matings occurring on the cage screen or on the floor should not be taken into account when computing the indices of mating performance given thereafter.

The following material is required:

- Plastic pill vials, scintillation vials, or similar containers, preferably clear (80 per cage).
- Grease pencils and/or masking tape and pens for marking vials.
- Long-wave ultra-violet lamp (should flies not be marked with paint and identifiable only by the presence of fluorescent dye) or fluorescent microscope.
- Small bottles of water-based paint; up to 4 light colours, to mark flies (should immediate identification of adult flies be needed and should flies not be marked only by fluorescent dye). Avoid using enamel paints that often contain mineral spirits that may influence the survival or behaviour of the insect.
- Thin, soft camel hair brushes for marking flies with paint.
- A minimum of 4 dental cotton wicks impregnated with water should be placed per tree as source of water for the flies.

Should additional tests be undertaken, the following material may be required:

- Dissecting microscope (only if females need to be dissected after mating to assess effective sperm transfer).
- Agar egging balls or ripe host fruit for an oviposition substrate; host fruit must be free of tephritid eggs. An average of one fruit or oviposition device should be available for every 20 females in the cage.

Procedure

At least 24 to 48 hours before the test, flies are marked individually according to their strain (wild or sterile) by applying a small dot of paint on the dorsal surface of the thorax. Immobilize flies by chilling them at approximately 5° C for a few minutes or by placing them in a bag made of mosquito net (18 mesh), placing the bag on a table, 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. This procedure is illustrated in **Figure 10**. Even though marking of flies with water-based paint appears to have no effect on mating performance field cage test results, colours used for marking strains should be randomised among replicates and brands of paints 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 in groups of 50 flies per 1 litre container (flies are provided with adult food [protein:sucrose 1:3], moisture, and ventilation in the containers) (see **Figure 11**).

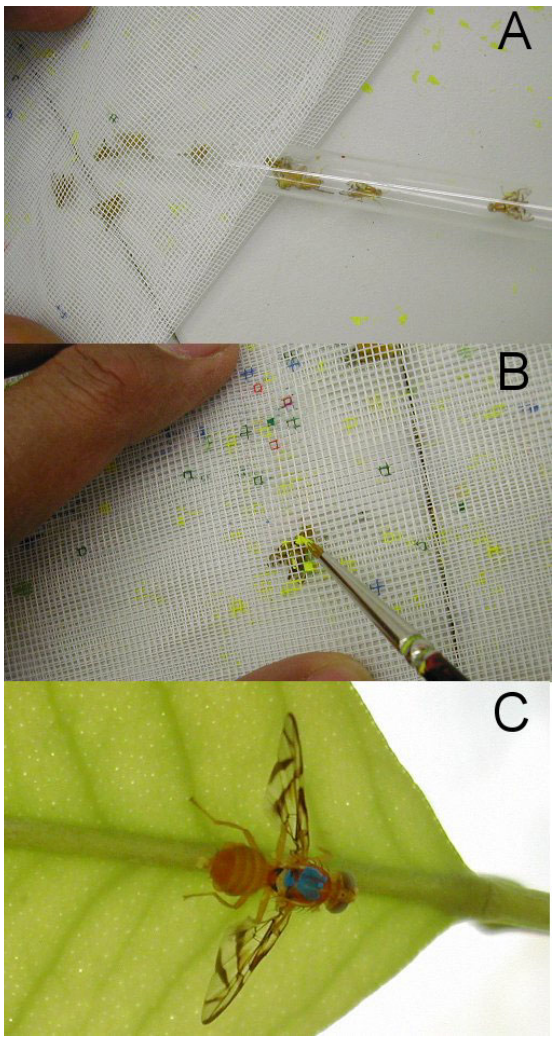

Figure 10: Procedure for marking tephritid adult individuals with water-based paint. *A. adults are gently blown from the aspirator into a bag; B. the bag is stretched to immobilize the fly and a drop of paint is made on the notum; C. the result: a marked Anastrepha fraterculus male calling in a field cage.*

On the day of the test, 50 wild and 50 sterile males are released into a screen cage and given a period of time (e.g., 15-30 min) to disperse and establish territories. Time of release should precede the time of peak mating for that species. Adults of *C. capitata*, for example, are typically released around dawn. 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 50 "normal" flies per strain per sex are maintained. Then, 50 wild and 50 sterile females are released.

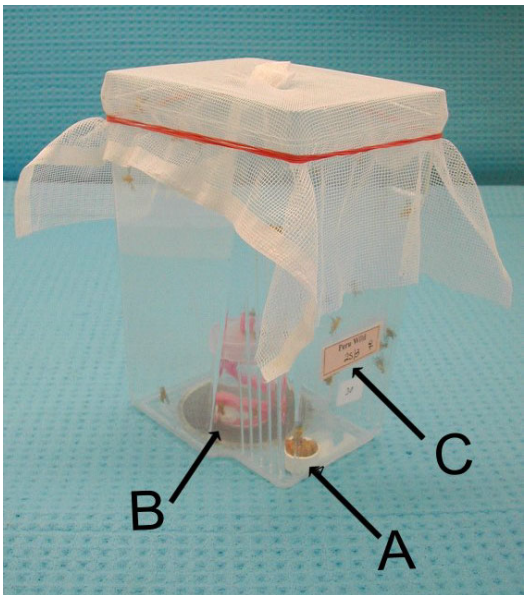

Figure 11: Container used to hold the flies after individual marking and before releasing into the field cage. *A. food (protein+sugar); B. water; C. label mentioning the strain, sex and number of adults.*

Starting time of mating performance field cage test should be adjusted according to environmental conditions and fly activity (i.e. early start on hot days) and test duration should cover most of the known sexual activity period for the species under the local conditions. In the absence of such information, the test should cover most of the day.

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

Figure 12: Collection of an Anastrepha fraterculus mating pair during mating performance field cage test

Census frequency should be at least every 30 minutes, or even shorter for species that have shorter mating durations. Capture mating couples individually in the vials, taking care to get only one pair (and only 2 flies) per vial (look at the pair carefully (**Figure 12**); males sometimes mount males and closely resemble a mating couple). If you lose one fly of a pair, release the other. If you have three, remove the extra (usually male) fly. Do not put two pairs in the same vial. If flies are not marked with paint but with fluorescent dye, keep them for a later recognition under the UV lamp or the fluorescent microscope.

Write time when pairs were captured on vials and other data such as an indication of position on the tree (height, substrate, etc.). Continue the test until the natural period of peak mating for that species under local conditions is well over. Record all pertinent data, including time and type of male and female for each vial, on the form given in 7.5 MATING PERFORMANCE ASSESSMENT FORM. 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.

When duration of mating is being recorded or the test is being run for more than one day, care must be taken to ensure that the vials are kept in the shade and otherwise handled to minimize thermal or other stresses to the flies.

***Notes on mating performance field cage test with genetic sexing strains (GSS)*:** Use 50 females from the rearing colony stock (not thermally treated) for tests of sexual compatibility. Additional tests should be run to evaluate overall sexual competitiveness of GSS males in the absence of GSS females. Data from tests that include *tsl*-GSS females may be unreliable if temperatures within the field cage are high (i.e., >27° C). In such cases, it may be acceptable to run only the "males only" cages (i.e., without GSS females). If mating performance of GSS males with wild females in "males only" cages is poor, the primary cause will need to be identified in sexual compatibility tests before corrective actions can be taken.

Optional Procedures for Mating performance field cage test

In addition to the standard procedure described in the previous paragraphs, it is recommended to collect the following information:

a) ***Duration of mating:***

Observe mating couples in vials on a regular basis (every 5 min or less) and note the time when uncoupling occurred. If these data are to be taken, special care should be taken when collecting mating

pairs to ensure that they are not disturbed to the point where they uncouple prematurely. Similarly, vials containing the couples should be oriented so that they do not have to be handled in order to observe the flies within them.

b) ***Male calling time:***

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 colour of mark are recorded for each male that is "calling", or releasing pheromone within the cage or involved in courtship with female. Also see "Pheromone Compatibility Test" in APPENDIX C: ANCILLARY TESTS.

Calling males are typically (but not always) on the underside of leaves. In medfly 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). In other species male 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 is more difficult to observe. These data can also be used to check diel periodicity of sexual behaviour among sterile males in comparison with wild males.

c) ***Incidence of remating:***

This requires continuation of the test through a second day. 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.

At the end of the first day's observations, flies are marked or remarked (use the mesh bag technique above, releasing one pair at a time into the bag). Marks should be colour-coded to denote whether the male and female mated previously with a wild or sterile fly. The flies are then released back into the cage, and the observations, as noted above, are continued for a second day. 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.

Should such a test be run, by no means should the flies be left inside the cage overnight. Uncontrolled changes in fly numbers due to flies being killed during the night by predators such as spiders, ants or environmental conditions could biase the results. Once collected from the cage, unmated flies should be sexed and held separately until they are released again on the next day.

d) ***Fried test:***

The Fried test is an excellent indicator of overall competitiveness of sterile flies in a field cage context. It's possible to run the Fried test as an extension of the mating performance field cage test, but, logistically, it is probably simpler to set it up separately. This test is covered in detail under "Fried Test" in APPENDIX C: ANCILLARY TESTS.

Interpretation

a) ***Overview:***

For meaningful data, the basic (first-day) portions of the mating performance field cage test should be replicated ca. 10 times. Test replicates with more than ca. 25% of wild flies on the cage screen during calling periods reflect inadequate environmental conditions (such as inadequate light, lack of water, leaves, etc.) and should therefore be repeated. Mean percentage of males and females mating from each strain provides a useful indication of mating propensity. With *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 flies from any combination of strain and sex participate in mating. Fly activities occurring away from the host tree should not be included in the data analysis.

Mean 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 only to monitor male sexual competitiveness, should be computed. Values of *SW* that are low compared to other possible combinations suggest that wild females tend to reject the courtship overtures of a high percentage of sterile 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.

b) ***Indices of strain sexual compatibility:***

Several indices have been developed to quantify the sexual compatibility between strain/population. The indices should be computed separately for each cage (based on the first day's data, if individual

tests are extended longer than one day). Analysis of test results should involve the use of the all of the main indices available (RII, ISI, FRPI, MRPI) and non-parametric statistics. They should also be presented in graphical form as shown in 7.6 GRAPHIC REPRESENTATION OF INDICES OF MATING PERFORMANCE). Data from mating pairs collected on the screen of the field cage or on the floor should be computed separately. These comments also apply to the index of male sexual competitiveness.

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 control; values greater than one indicate positive assortative mating, i.e., steriles tend to mate only with steriles and vice versa (see **Figure 13**). 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 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 sterile females accept wild and sterile males equally and that wild and sterile males will have equal mating propensity in the open field, then the *RII* is equal to the number of sterile 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.

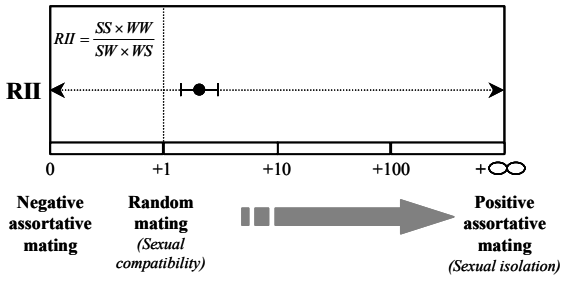

Figure 13: Graphic representation of the Relative Isolation Index (RII). *The value shown represents the mean value obtained when comparing wild and sterile Ceratitis capitata flies in field cages.*

RII also has several disadvantages. First, it is undefined if *SW* or *WS* is 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.

Values of *RII* larger than 1 indicate that there is some difference in mating behaviour (in a broad sense) between wild and sterile flies, and that one or both strains are 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 strains of sterile flies). For *C. capitata, RII* has typically averaged from 1.5 to 5. For mating of sterile flies from the Hawaiian Hi-Lab strain with SIT-"resistant" *C. capitata* on Kauai, *RII* ≅ 30. In general, values of *RII* consistently larger than 3 for *C. capitata* suggest that rearing facilities should start considering alternative strains for production.

Stalker's Index (I). The Stalker's Index (I) is similar to the reciprocal of *RII* except that the terms in the numerator and denominator are added rather than multiplied.

$$I = \frac{SW + WS}{SS + WW}$$

This makes it more stable to changes in a single type of mating, but less sensitive to specific drops in the critical *SW* type of mating. In addition, *I* is affected somewhat by how many of each type of fly participate in mating; that is, it is affected by mating propensity or the ratio of *S*:*W* in the cage as well as mating compatibility.

Values of *I*, like those of 1/*RII*, extend from 0 (total isolation of the two strains) through 1 (random mating) and on to infinity (all matings with members of the opposite strain). This index is probably the most commonly encountered index of mating compatibility. It has been cited on a number of occasions in reference to fruit fly quality control, but also appears in more basic studies of interbreeding among different strains, populations, and closely related species.

Isolation Index (ISI). The *ISI* is a 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) (see **Figure 14**). 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 the case of *C. capitata* values of *ISI* usually range between 0.1 and 0.4. In general, values of *ISI* consistently larger than 0.5 suggest that some assortative matings took place (which should be explained by analysing the values of *MRPI* and *FRPI*) and that replacing strains should be considered.

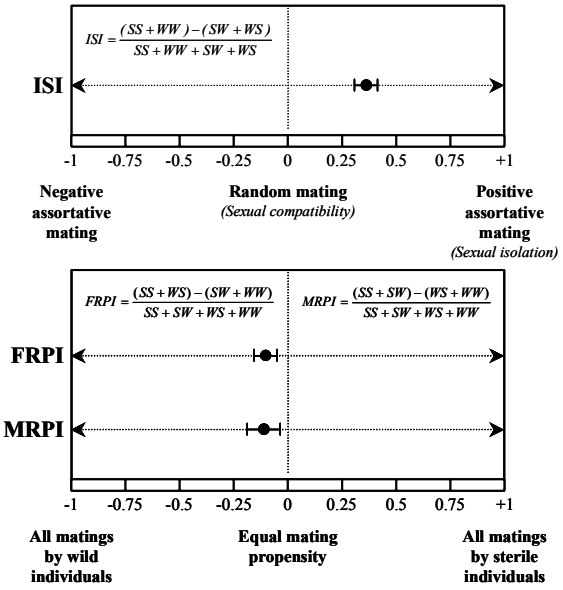

Figure 14: Graphic representation of the Isolation Index (ISI) and of the Male and Female Relative Performance indices (MRPI and FRPI). *These indices should only be considered together for a better understanding. The value shown was obtained when comparing wild and sterile Anastrepha ludens flies in field cages (after Hernandez et al. 2003).*

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

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

A value of 1 indicates all matings in the cage were done by sterile males, and -1 indicates all mating was done by wild males. Zero indicates that wild and sterile males participated equally in mating. (see **Figure 14**). This index must be used in addition to the mean percentages of different types of flies participating in mating, and complements the *ISI* and *FRPI* (below).

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 (see **Figure 14**).

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

The joint analysis of *ISI*, *MRPI* and *FRPI*, should provide a complete and reliable picture of the sexual compatibility between strains and, should a deviation from the expected standard be encountered, the reasons why it occurred. In general, when comparing a mass-reared strain and a wild population of *C. capitata*, ISI values range between 0.1 and 0.4, FRPI values are positive values and MRPI values are close to 0. These data show that, in such a case, there is a slight tendency toward assortative mating, that sterile 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.

c) ***The Relative Sterile Index (RSI), an index of male sexual competitiveness:***

The *RSI* is the major index of 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 (see **Figure 15**). 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.

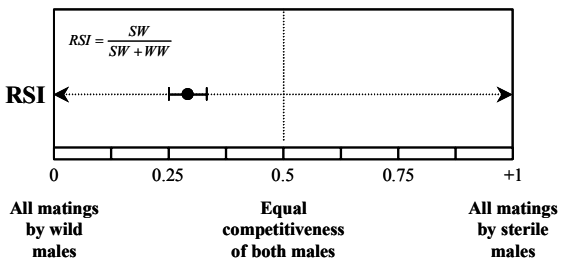

Figure 15: Graphic representation of the Relative Sterility Index (RSI). *The value shown was obtained when comparing wild and sterile Anastrepha ludens flies in field cages (after Hernandez et al. 2003).*

d) ***Median time of copulation:***

If the timing of mating appears to be different for sterile versus wild type flies, the median time of mating, or H_{50}, can provide a useful index to quantify mating periodicity.

$$H50 = \frac{\sum_{i=1}^{n} D_i}{\sum_{i=1}^{n} n_i}$$

Median time of mating for each type of fly (sterile male, wild female, etc.) is computed separately for each cage. Periodicity of mating can then be compared in pairs (e.g., comparing H_{50} for wild versus sterile flies) using paired *t*-tests.

e) ***Duration of mating:***

Length of time spent *in copula* can be an indication of laboratory adaptation of a strain and can be related to transfer of sperm and accessory gland fluid to the female fly.

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, short duration of mating for sterile males, relative to those of wild males mating with the same type of female, is reason for concern and may correlate with other data, such as those on the incidence of remating. 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.

f) ***Male calling time:***

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 or 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. The ability of males to attract unmated females can be assessed using the ancillary "Pheromone Compatibility Test" described in APPENDIX C: ANCILLARY TESTS.

g) ***Female acceptance:***

Estimating the rate at which females accept sterile or wild males requires more detailed observational studies, quantifying the number of female visits to the different types of calling and courting males, and recording rejection or acceptance of males by females.

h) ***Incidence of remating:***

Remating in wild tephritid females is more common in nature than previously believed. Sterile males are in general less able than wild males to suppress remating in wild females. 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.

Modification to the Mating performance field cage Test

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 medfly 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.

3.2. Release-Recapture Tests 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. Data from these tests are useful for making comparisons between different rearing strains or among flies from different facilities.

Aside from assessing fly quality, an important function for these tests is to determine if the release protocol being used in the operational programme provides continuous coverage throughout the programme area. Data from these tests can be used as a basis for adjusting operational procedures, such as the time interval between two releases in any given area or, with appropriate modification, the distance between release points (or flight lines).

During ongoing SIT programmes, these tests should be run in an area that is close enough to the programme release zone that climate and habitat will be similar. As an alternative, release-recapture tests 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.

Equipment and Supplies

- Attractant-baited traps, appropriate for the species being tested (see below).
- Dissecting microscope.
- Long-wave ultra-violet lamp.
- Screen cages.
- Miscellaneous cups, forceps, or other supplies as necessary.

Procedures

Sterile, dye-marked insects are shipped to the release facility and handled according to standard 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, if possible, making it possible to follow individual releases for multiple weeks. 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. 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*. 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*, McPhail traps baited with 5 Torula yeast pellets each are still considered the standard.

Traps are hung at the trap sites 1, 4, and 7 days after each release, and, in each case, are then 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. The following guidelines should be considered:

- Traps should be placed in a regularly spaced grid of at least 6 x 6 traps. A series of concentric circles of traps is generally considered better than a grid for measuring dispersal, but circular plots are often much more difficult to lay out and check. In addition, use of a regular grid ensures that trap density will be the same throughout the test plot.
- 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. Flies within the cages should be protected from predation and provided with abundant food (typically 1:3 yeast hydrolysate:sucrose), water, and shelter from direct sunlight and rainfall. Walk-in field cages containing live plants for shelter may be used if it is possible to exclude crawling predators such as spiders and ants to a sufficient degree. As an alternative, small cages (e.g., 30 x 30 x 30 cm) may be hung from trees or placed on tables. In either case, small cages should have solid roofs for shelter, and they must be protected from predators such as ants. Any vegetation touching the cages must be removed, and Tanglefoot or a similar sticky substance must be applied to any hangers, table legs, etc., 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 cages (separate cages for each strain) at the time of release and are then checked for mortality whenever traps are checked. 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 1 days after being released versus 3, 6, 8, 10, and 13 days after the release provide estimates of survival for these periods of time out in the field. 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 and 8 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. 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 of 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 Ci is the number of flies captured in trap i, and xi is the distance between trap i and the release point.

3.3. Relevant Literature

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