12. Adult processing and identification of recaptured sterile flies

STEP VI OF PROCESS IN FLOW CHART IN APPENDIX 2

12.1 OBJECTIVE

The objective of this process is two fold: (1) monitor sterile fly densities, and (2) identify fertile/sterile flies. The first provides feedback on the effectiveness of the release procedures in attaining the desired sterile fly density and sterile:wild ratio. The second is important in evaluating the effectiveness of the release in terms of reducing or eliminating fertile fly populations and also when the identification of fertile flies is a trigger for implementing suppression or eradication procedures.

12.2 TRAPPING SYSTEMS AND COLLECTION PROCEDURES

Released sterile flies are re-captured in the same traps that are used for detection of the wild population. For example, the most common trap and lure types used for Medfly detection are Jackson traps with a male specific lure (Trimedlure) and Multilure traps with a female biased lure (Biolure) (**Figure 12.1**). The densities of the traps vary depending on the programme objectives. Trapping recommendations for this fly species and others are detailed in Trapping Guidances for Area-Wide Fruit Fly Programmes (IAEA 2003), although some trap types, such as the Lynfields (Cowley *et al.* 1990, Dominiak *et al.* 2003) used in Australia, are not included in this document.

In an area subjected to sterile fly releases, the vast majority of flies caught will be sterile. Typically the flies are collected during the normal servicing of traps and brought from the field at the end of each day. When wet traps (e.g. Multilure and McPhail) are checked for fly catches in the field, samples are stored in a suitable preservative solution such as 70% isopropyl alcohol. In the case of dry traps such as Jackson or Open Bottom Dry Trap (OBDT) flies caught are left on the sticky insert and transported to the identification centres. In general flies are examined the following day.

FIGURE 12.1
Jackson (left) and Multilure (right) traps (Courtesy CDFA).

12.3 MARKING SYSTEM

When identifying sterile insects it is important to have a rapid method of distinguishing them from fertile flies. The only marking system currently used for fruit flies involves the application of a fluorescent dye onto the surface of the pupae, which is then transferred to the teneral adult upon emergence. Steiner (1965) first reported this method of identifying large numbers of released sterile flies. This method initially used oil soluble dyes and required crushing the head and extracting the dye. Holbrook *et al.* (1970) reported on the use of fluorescent dyes and the use of ultra-violet light. This was subsequently improved to increase the accuracy of discriminating between unmarked wild and dye-marked released flies (Enkerlin *et al.*1996).

The amount of Day-Glo dye applied to Medfly pupae ranges between 3.0 – 4.0 grams per kg of pupae (1.5 – 2 grams per litre of pupae). This dose may vary depending on the fly species and the needs of the individual programme. Many dyes are hydrophilic and excess amounts of dye may cause dehydration of pupae: higher body weight is an important parameter contributing to other quality parameters (Dominiak *et al.* 2002). Some dyes contain levels of deleterous chemicals such as formalin. Most dyes are manufactured in different particle sizes with smaller particles potentially clogging respiratory passages (Dominiak *et al.* 2000, Weldon 2005) A crucial element to this process is to have the pupae clumped together to increase the amount of dye covered surface area with which the emerging flies come into contact. Various colours are used depending on individual programme preferences, with the most commonly used one being red-orange. The dyes are visible under white light as dull colours, but they become most visible when viewed under ultraviolet light because the dye colour brightens and fluoresces. Some dyes reduce emergence or may interfere with dispersion ability and recapture rates (Dominiak *et al.* 2000, 2003, Jackman *et al* 1996).

The most useful area to find dye on the fly body is a membranous pocket in the head capsule called the ptilinum. This membrane is used by the fly during emergence from the pupa to break open the hardened puparium surrounding the pupa. Haemolymph is pumped into it to force it out of the head and enlarge it to an extent that it breaks the puparial shell. Shortly after the fly has emerged from the puparium, the ptilinum is retracted back into head and is not exposed again for the life of the fly. During the brief period when the ptilinum is exposed it typically becomes covered with dye dust from the outside of the puparium. Unlike the other parts of the body, the amount of dye on the ptilinum does not decrease as the fly ages because it is withdrawn within the head capsule (**Figure 12.2**).

FIGURE 12.2
Freshly emerged adult fruit flies with pink dye clearly showing in the ptilinum. It is essential for dye to be retained in the ptilinum to ensure correct identification of wild and sterile flies.

Research is ongoing to develop genetic and biochemical markers which confer identifying characteristics, such as bioluminescence or pigment changes, to the flies, but none of these are currently used in operational programmes (for more information see Sections 12.7 and 12.8).

FIGURE 12.3
Ultraviolet light and dissection microscope combination for examining dyed fruit flies (Courtesy USDA and FDACS).

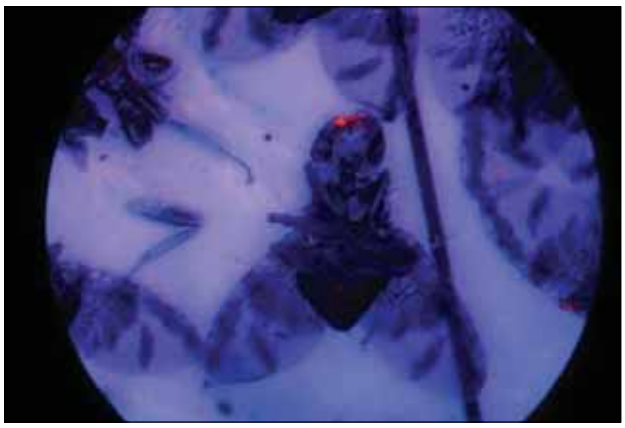

FIGURE 12.4
Sterile *C. capitata* showing marking dye on ptilinum underneath ultraviolet light (Courtesy USDA and FDACS).

12.4 INTERPRETATION OF STERILITY VIA EXAMINATION FOR DYE PRESENCE

The dye is most visible when viewed under ultraviolet light in a darkened environment. A current setup used in the Florida Preventive Release Programme uses an ultraviolet ring light attached to a dissecting microscope (**Figure 12.3**). The dye can be observed either on the external surface of the fly and/or on the ptilinum within the head capsule (**Figure 12.4**). Flies displaying the dye in the manner detailed below are considered to be sterile. In Australia, a blue light is used in a darkened room to minimise any health concerns associated with ultra violet light. If no dye is found under blue light, then flies are examined under ultra violet light.

Dye in the ptilinum is the definitive method to determine if flies are sterile and programmes should aim at 99.5% of recaptured flies having dye in the ptilinum. Dye on the ptilinum can often be seen around the edges of the ptilinal fissure and through the frons. In those instances when it can not be reliably seen, the ptilinum can be exposed in one of two ways. One is to mimic the original method of exposure, namely forcing liquid into the ptilinum. This can be accomplished by collapsing the head capsule, thereby forcing liquid forward into the ptilinum. The easiest way to do this is to lay the head on its side and gently press down. The ptilinum can also be exposed by using forceps to press against both eyes. This latter method should not be used in conjunction with examination for dye with a white light because reddish retinal tissue exiting the eyes can be mistaken for reddish dye. Alternately, the ptilinum can be physically pulled out of the head capsule by pulling on the frons just below the antennae.

Ptilinum dye is the most reliable method of identifying sterile flies. However a small number of flies may not pick up dye in the ptilinum and other tests must be used to subsequently determine if flies are wild or poorly marked sterile flies. Other parts of the body must be examined for the presence of dye. Dye that initially adheres to hardened surfaces is more likely to be removed prior to recapture than dye that collects on softer membranous surfaces. Exposed dye gradually falls off, is removed by flies during grooming, and/or can be washed off in a liquid based trap. A caution is that dye can also transfer from one fly to another in a trap on these exposed areas. Therefore, the best areas to look for dye on the fly's body are on membranous areas between the sclerotized portions of the body, especially underneath the wings and at the leg joints

and in the base of the neck. The most reliable pattern of dye is a scatter pattern similar to that made by the discharge of a shot gun. Dye in these body crevices is not easily preened off by flies and is a reasonable secondary indicator.

The collection method used within a trap can contribute to transfer of dye from a sterile fly to a fertile fly. For example, traps using sticky boards that rely on the sticky substance to entrap and kill the flies can have dye embedded in the sticky substance because captured sterile flies may struggle for some time before death. Dye that has become embedded in the sticky substance can then potentially be transferred to fertile flies subsequently landing next to the entrapped sterile. In addition, traps using liquids as a killing agent result in dye particles washing off into the liquid which can potentially be transferred to fertile flies caught in the same trap. In dry non sticky traps such as Lynfield traps, malathion causes the dying flies to buzz and convulse. These actions may cause some dye particles to be transferred to a wild fly. Therefore a high level of ptilinal dye is important to confidently and accurately identify sterile flies.

Some programmes process the flies prior to dye examination (Enkerlin *et al.* 1996, Programa Regional Moscamed 2003). In these programmes the flies are removed from the trap and placed onto a gridded sticky board. The flies are then examined for dye. For those flies where no dye is seen on the external surface, the heads are removed and placed onto a similarly gridded sticky board, crushed and then examined for dye. Some programmes only look for dye on the ptilinum (Guillen Aguilar 1983), in which case all heads are routinely taken off all of the flies, lined onto gridded paper, crushed, and examined for dye. Acetone has been used to wash the dye from the head but results may be variable for different dye formulations.

To increase accuracy in discriminating between sterile marked flies and wild flies an epi-fluorescent compound microscope (Nikkon Model Y2B-EFD-3, 1990; objective CF ACHRO 10, 20, 30, 40 and 100x; oil iris diaphragm; fluorescent filter B-24 and Epi-fluorescent accessories EPI-FIELD) can be used (Enkerlin *et al.* 1996). The epi-fluorescent microscope is more powerful than the conventional ultraviolet lamp normally used. The amount of dye used to mark the flies can be reduced if a more powerful tool for detecting marked sterile flies such as the mentioned microscope is available. Excess dye in the sterile flies has substantial detrimental effects on quality including survival and flight ability. Weldon (2005) reported that light wavelength was an important contributor to making dyes more visible, with a light filter in the blue range (467 nm) being optimal for the dye evaluated. Some programmes use different colours to evaluate different treatments. However some colours such as Deep Green and Chartreuse were highly visible under blue light but not visible under green light (511 nm) or yellow light (563 nm). Lilac was more visible under green and yellow light but less visible under blue light. Programmes need to carefully match their laboratory identification services with the dyes used in the field.

12.5 INTERPRETATION OF STERILITY VIA EXAMINATION OF REPRODUCTIVE ORGANS

Flies dislike the dye particles and expend energy to preen dust from their bodies and wings. Excess dye may result in excess preening and subsequently low energy for searching for food and shelter. The determination of the sterile/wild status based on ptilinal dye is quick and cost effective. Using other techniques, such as examining the deep body crevices for dye, using DNA, or examination of sperm (all described below) are much more time consuming and expensive. They should be used only after the examination for ptilinal dye has failed to detect dye.

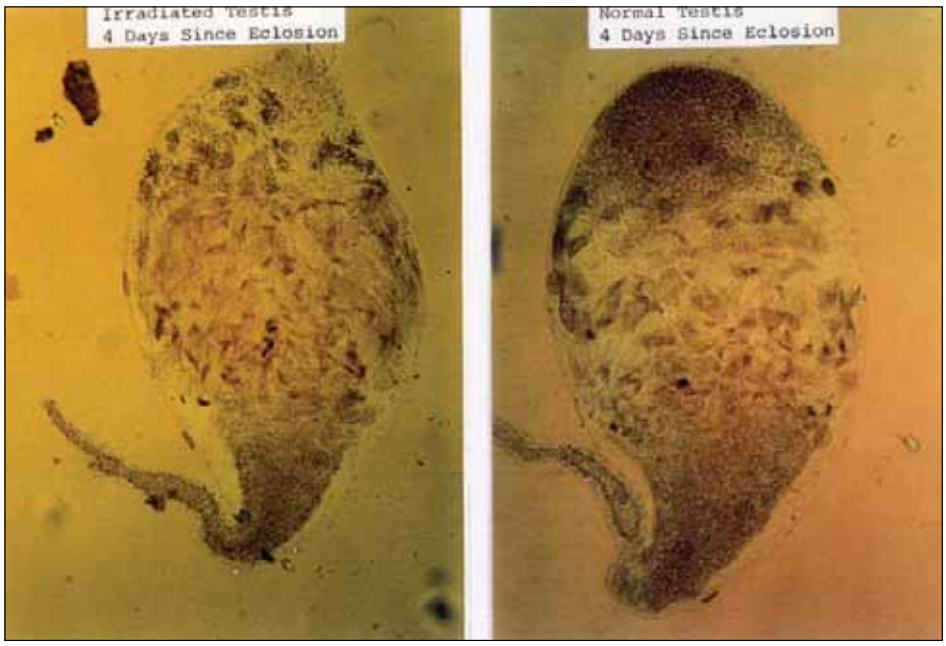

FIGURE 12.5
Testes from irradiated (left) and non-irradiated (right) male *C. capitata* four days post emergence (Courtesy CDFA).

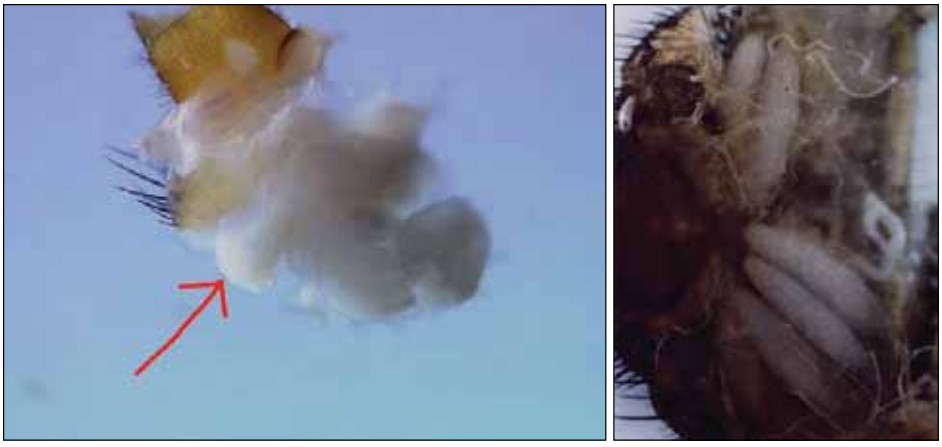

FIGURE 12.6
Ovaries from irradiated (left) and non-irradiated (right) sexually mature female *C. capitata* (Courtesy CDFA).

Occasionally recaptured flies will show no definitive evidence of having been dyed. In these cases the reproductive organs can be examined to determine whether the fly had been irradiated (Guillen Aguilar 1983). Typically, the percentage of irradiated flies requiring this level of examination should be extremely low, e.g., in the neighbourhood of 0.004% of the recaptured sterile Medfly for the California PRP.

The damage to the reproductive organs caused by irradiation results in a cessation of sperm and egg production by killing the reproductive cells. In males, this damage occurs after some sperm is already in production, so an irradiated male will have a certain amount of sperm. However, the DNA in the sperm is damaged so that the fly is functionally

sterile. Production of new sperm is prevented by the death of the reproductive cells. In females, irradiation prevents the production and maturation of eggs**.**

The damage to the reproductive cells can be seen by microscopic examination. For males, the testes must be slide mounted and examined in a 2% aceto-orcein dye under a compound microscope. Female ovaries can be examined directly beneath a dissecting microscope. An excellent day-by-day chronology of development in irradiated and non-irradiated Medfly can be found in the work by Guillen Aguilar (1983).

The testes of a sterile male show a progressive deterioration with age. The germarial cells die from the bottom to the tip. The cells undergo pycnosis where they collapse into themselves. This is seen visually by numerous dots surrounded by empty space (**Figure 12.5**). Sperm may migrate up into the germarium through these spaces. Also, spermatid production is halted so the characteristic "strawberry"-shaped spermatids disappear. In contrast, the germarium of fertile flies consists of closely packed, well defined cells and the spermatids are present in the zone below the germarium.

The ovaries of a sterile female are present as translucent sacs that can be examined visually without slide mounting (**Figure 12.6**). The ovaries of fertile flies have eggs in various stages of development.

12.6 INTERPRETATION OF FEMALE MATING STATUS VIA EXAMINATION OF REPRODUCTIVE ORGANS

While not an indicator of irradiation exposure, it is often of use to programme managers to determine the mating status of captured fertile females. This is accomplished by removing the spermathecae and slide mounting them in aceto-orcein. The spermathecae are then crushed by gently pushing down on the cover slip. This exposes any sperm inside, which then can be seen as a tangled mass in oval spermathecae from *Anastrepha*

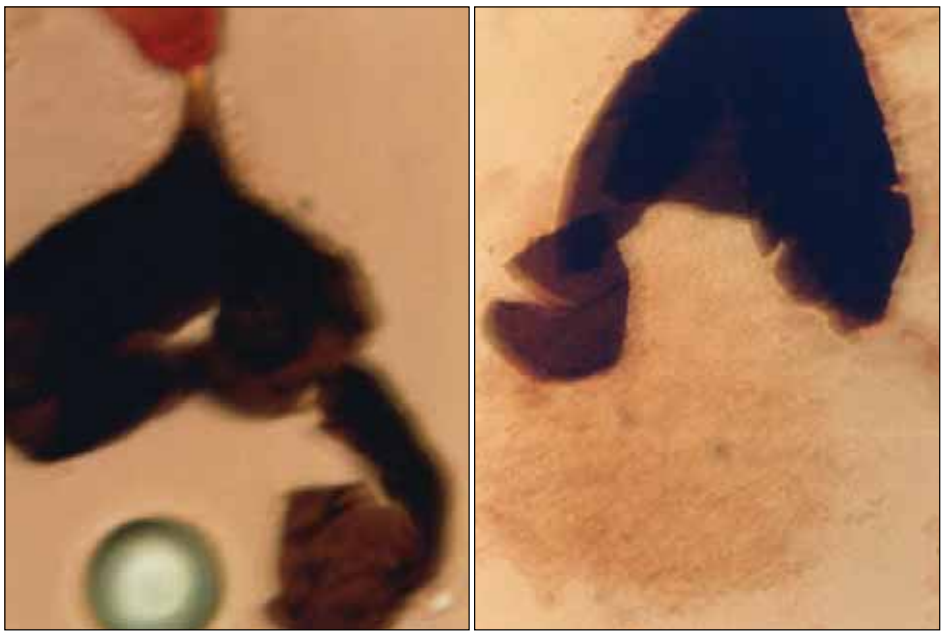

FIGURE 12.7
Spermatheca from unmated (left) and mated (right) female *C. capitata* **(Courtesy CDFA).**

and *Ceratitis* (**Figure 12.7**) or as an ordered rope-like mass in coiled spermathecae from *Bactrocera* (**Figure 12.8**).

A question often asked by programme managers is whether a mated fertile female was mated with a sterile male. This is useful information because it provides feedback on the success of the releases and it can identify localities that may require more intensified efforts. Currently, this can only be answered to some extend for one species, Medfly, by using a method of sperm head measurement (McInnis 1993). Research is underway to develop genetic and/or biochemical sperm markers to address this problem, but none of these systems have been incorporated into large scale release programmes.

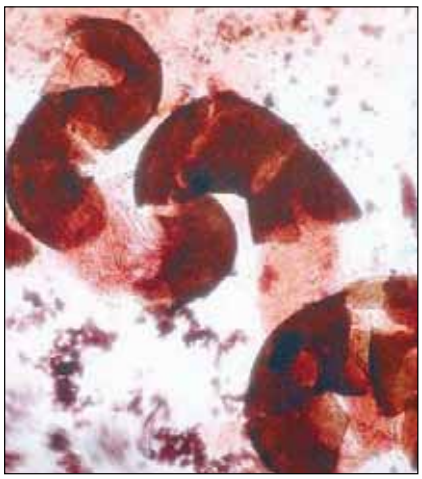

FIGURE 12.8
Spermatheca from mated female *Bactrocera oleae* (Gmelin) (Courtesy CDFA).

12.7 MITOCHONDRIAL DNA ANALYSIS

Molecular markers can be very useful in order to differentiate released flies from wild flies and endogenous populations from invasions. A class of markers that are in use for Medfly are derived from the mitochondrial DNA (mtDNA) molecule using restriction site analysis. In order to identify a marker in a release strain it is important to know the genetic variation (haplotypes) in this molecule in the target field population (IAEA 2004). The same applies to populations from different geographical regions for which haplotypes can be determined and used to compare with those of endogenous populations. This technique is routinely being used in some prevention programmes to identify possible sources of incursion of exotic flies (Yu *et al.* 2001, Sved *et al.* 2003) and in others it has been used to provide assurance to programme managers that unmarked fly finds are not unsterilized flies from the mass rearing facility (Barnes *et al.* 2004).

Another approach to marking flies for release is to transform them with a genetic construct that expresses a fluorescent protein in different body parts or in the sperm. A genetic construct has been introduced into the Medfly VIENNA 8 genetic sexing strain for evaluation (IAEA 2004).

12.8 MORPHOLOGICAL MARKERS

A dominant mutation called *Sergeant* Sr^2 could be used as a visible marker for Medflies released into the field. This mutation has been incorporated into the VIENNA 8 only male strain. The marker consists of three abdominal bands instead of the two bands that the normal medfly strain has. Releasing Medfly only male strain with the addition of a visible marker would very much simplify the discrimination between sterile released males and wild males caught in traps. Initial work on the fitness of the mutation in terms of mating behaviour showed no negative effects on the strain carrying the mutation. Furthermore, the VIENNA 8 strain with the visible marker has very similar quality profile compared with the normal VIENNA 8 strain (Niyazi *et al.* 2005). This is very encouraging for any eventual use of the strain in an operational SIT programme. However, an open field evaluation should be conducted before any decision is made on the use of the strain.

This will have to include a trapping component to assess if the marker is useful when the sterile flies are trapped and exposed to weathering in the field (Robinson and Hendrichs 2005).

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