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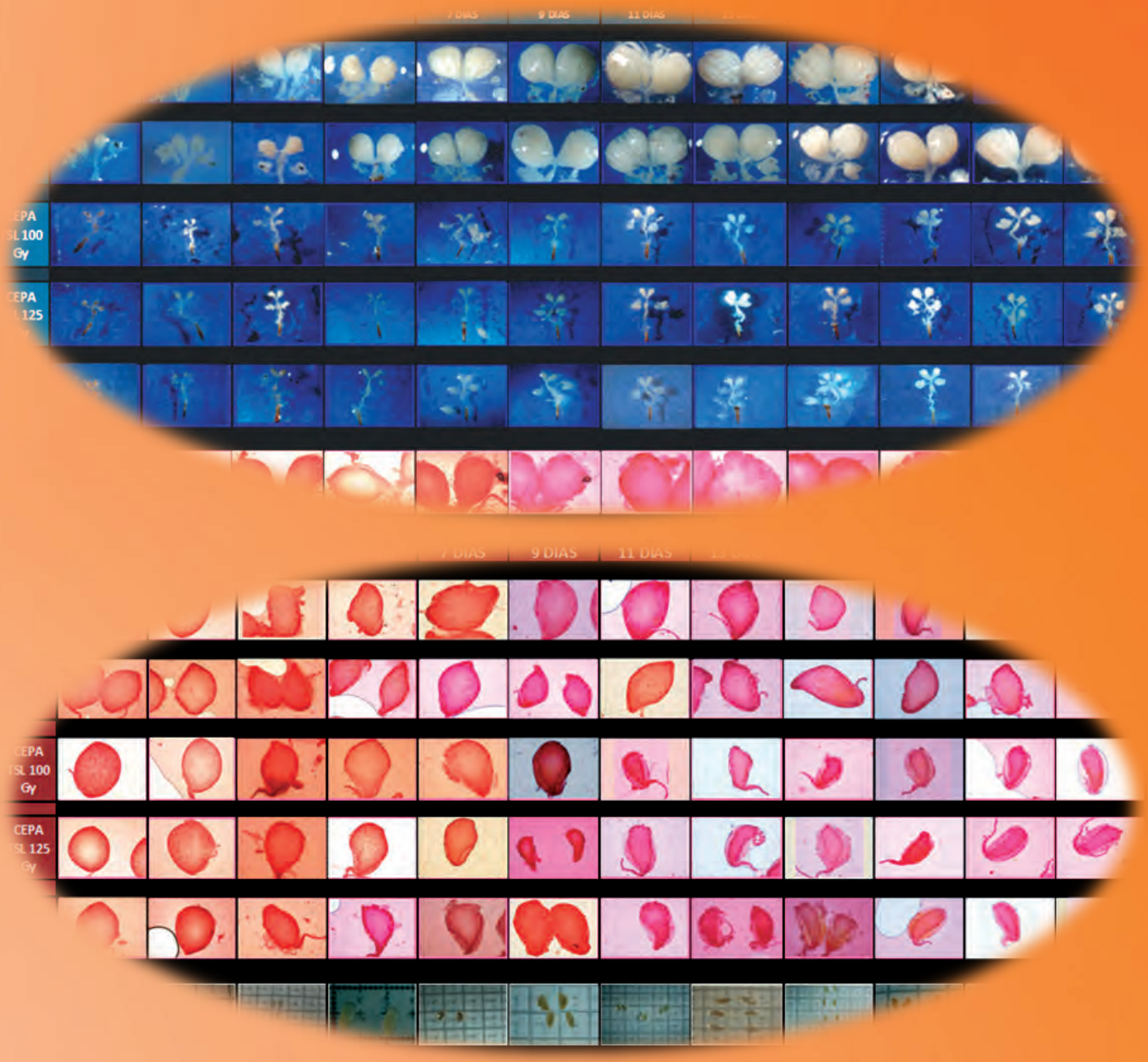

Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

Manual to Differentiate Wild Mediterranean Fruit Flies *Ceratitis capitata* (Wied.) from non-irradiated (Fertile) and Irradiated (Sterile) VIENNA Temperature Sensitive Lethal Strain Flies

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Foreword

An important component of the implementation of the Sterile Insect Technique (SIT) is the induction of dominant lethal mutations to achieve sterility in released flies and the subsequent identification of the different flies captured in traps. The accurate identification of wild and sterile flies is central to insect pest management programmes that integrate the SIT as well as to the application of other appropriate pest control strategies.

In the specific case of the Mediterranean fruit fly, *Ceratitis capitata* (Wied.), due to its major quarantine and economic importance, misidentification of flies may have serious consequences. Identifying sterile flies as wild flies in areas where sterile flies are released to suppress or eliminate wild populations may lead to implementation of unnecessary mitigation measures that can be time-consuming and costly. Identifying wild flies as sterile flies may cause premature suspension of control actions in areas with small residual populations which can put at risk the progress of suppression and eradication programmes.

This manual is a product of recent studies on the effect of gamma radiation on testes and ovaries of the VIENNA Mediterranean fruit fly temperature sensitive lethal (TSL) genetic sexing strains, which are being used in most Mediterranean fruit fly mass-rearing facilities in the world. It includes standardised and updated procedures to determine the fertility or sterility of adults of these strains. Consequently, this is a very useful document to support SIT based area-wide integrated pest management programmes that release VIENNA Mediterranean fruit fly TSL strains.

These studies were partially funded by research project No. 15403 sponsored by the Insect Pest Control Section of the Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture.

The Programa MOSCAMED (SAGARPA-SENASICA) played an important role in these studies by providing funds and access to the Mediterranean fruit fly mass rearing facility, identification laboratories and to the International Fruit Fly Training Centre, both placed in Metapa de Domínguez, Chiapas, Mexico.

Contents

1. Introduction

The sterile insect technique (SIT) has been applied worldwide for 50 years to suppress or eradicate insect pests following an area-wide integrated pest management approach. Its application against the Mediterranean fruit fly (Medfly), *Ceratitis capitata* (Wied.), has been one of the most significant and successful uses of this technology.

The development of the *VIENNA* Mediterranean fruit fly temperature sensitive lethal (TSL) genetic sexing strain (Franz 2005), has improved the cost-effectiveness of mass rearing and sterile fly release methods. Because only males are produced and released in the field, costs are significantly reduced and any fruit damage caused by sterile females when attempting to lay eggs in the fruit is avoided. Most importantly, in the absence of sterile females, sterile males compete directly with wild males for mating with wild females without wasting their limited sperm on mating with sterile females. In contrast, the field releases of bisexual strains have higher rearing and release cost, and lower efficiency because the sterile individuals might prefer to mate with each other, thus reducing the chances of effective pairings between sterile males and wild females.

The changes produced by irradiation of late pupae in testicles and ovaries of adult Medflies are gradual and become more evident as they mature with increasing age. This process is well documented in the *"Manual for the differentiation of wild (fertile) Mediterranean fruit flies Ceratitis capitata (Wied.), from irradiated (sterile) flies"* prepared by Jorge Guillen (1983). For many years this pictorial manual has been a point of reference to determine the sterility or fertility of Medfly irradiated with 145 Gy two days before fly emergence, which used to be a standard irradiation procedure for bisexual strains.

Nevertheless, when the Programa MOSCAMED in Guatemala and Mexico started releasing the VIENNA Mediterranean fruit fly TSL strain, with the sterile males irradiated with 100, 125 or 145 Gy, one day before emergence, program staff using the above-mentioned manual began reporting inconsistencies between the images and descriptions in the manual and the observed features in the testicles of the captured TSL sterile males, causing some of the diagnoses to be questioned, especially in flies between 4 to 6 days old.

The differentiation between fertile and sterile flies involves the application of highly specialized procedures by well-trained technicians, which in first instance detect the fluorescent markings of the captured dye-marked sterile flies and then, in cases of unmarked flies, determine the sterility or fertility through a detailed cytohistological study (see flow diagram, **Appendix 1**). Therefore this manual will be a helpful reference for these skilled staff whose work is of major importance to SIT programmes.

2. Determination of the sterile or **fertile condition of adult flies through observation of dye markings**

2.1 Marking of adults with fluorescent dye

In Medfly mass rearing and sterilization facilities marking of flies is carried out at the pupal stage before irradiation. This procedure consists on impregnating the pupae with a limited amount of very fine fluorescent organic dye of plant origin (Day-Glo®) (Fig. 1a). Adults become marked with the dye during emergence as the dye adheres to the ptilinum when it is used by the fly to force its way through the end of the puparium. The ptilinum is a membranous and eversible pouch located above the base of the antenna, in the frontal part of the fly head. It retrieves back inside the head after emergence, thus, leaving the ptilinal suture (also called frontal suture), visible in the head.

Subsequently, the marked sterile pupae are irradiated. Once the adult sterile flies emerge, they are released in the field where they disperse and mix with the wild pest population. After Medfly adults are caught in the traps the process of differentiation between the released sterile flies and the wild fertile flies begins.

2.2 Arrival of trapped adults to the Identification Laboratory

The following procedures are mostly used for trapping networks set up with dry traps and sticky inserts. When servicing a trapping network the trapping staff removes the sticky inserts from the traps. All collected inserts are submitted to an identification laboratory where staff verifies and registers the number of inserts delivered (Fig. 1b).

Identification laboratories should have the necessary conditions and infrastructure to allow for an appropriate working environment, (e. g., ergonomic tables and chairs and air conditioning). They should include safety and sanitation measures required for activities that involve the use of chemicals. The laboratory should have separate areas to work in low and no light conditions. Because of the critical importance of the correct determination of the sterility or fertility of flies, appropriate materials and equipment should be available in the laboratory. **Appendix 2** provides a list of the most commonly used materials and equipment. The number of identification staff in the laboratory and skill level, should be adjusted according to the prevailing workload. This will reduce the probabilities of human error in the determination of fly sterility or fertility

2.3 Removal of flies from trap's sticky inserts

Sticky inserts with captured flies are removed with a wooden stick from the traps. The flies present on each insert are placed one at a time on a gridded sheet (25 x 16 cm) using disposable wooden sticks. The sticky insert code should be recorded in the first box (2 x 2 cm) of the gridded sheet, and the flies should be placed in the subsequent boxes (one fly per box). The data of the next sticky insert with captured flies is recorded in the next empty box of the same gridded sheet, repeating the process until no more inserts with flies are left (Fig 1c-d).

To prevent cross contamination, each end of the wooden stick should be used only once; after which the stick should be broken and discarded to ensure it is not reused. For easy observation each fly should be placed with the head facing upwards and towards the front in one of the boxes of the grid. Then, using a magnifying lamp or a simple stereoscopic microscope, the sex of each fly is determined and recorded

in the box of the grid using the symbol "♀" if it corresponds to a female fly. Boxes with no symbol mean the fly is male.

The sex determination of females is made by confirming the presence of the ovipositor on the tip of the abdomen. The last abdominal segments are modified to form the female terminalia which includes the sintergosternite 7 which has at the end an eversible membrane (oviscapt) and the ovipositor (aculeus) (Fig. 6).

The sex determination of males is made by confirming the presence of the male terminalia in the last abdominal segments, where the aedeagus is placed and observed as a thin copper-colored sclerotized rolled tube. Another important taxonomic feature in identifying males is the pair of frontal spatulate-shaped antenna located in the frontal zone of the fly's head (Fig. 2b).

2.4 First step: Observation of flies under ultraviolet light

Observation of flies is carried out in a dark room with ultraviolet (UV) light to detect the fluorescent dye marks. The UV lamps (Black Ray model B-100AP) have a light concentrator cone that enhances an incident point in the illuminated field. Therefore if a lamp like this is used the fly to be observed must be placed exactly on this point. The observation of flies can be carried out directly under UV lamps (Fig. 2a) or, for better observation; a stereoscopic microscope together with an UV lamp can be of help (Fig. 2d). Effective observation of marked flies will depend on the quality of the fluorescent dye marks, the intensity of the UV light and the darkness conditions of the area.

Some flies when captured in traps can become contaminated on their wings, legs and other body parts with particles of the dye markings of previously captured flies. It is important that these contamination marks are not considered for fly determination. To prevent these sorts of errors, the flies should be placed with the head in front of the observer so that he/she can easily distinguish the head frontal zone and the ptilinal or frontal suture (Fig. 2b-c) since the dye marks in the ptilinal suture is the most important feature to be observed (Fig 2f).

Flies with observable fluorescent markings should be determined as "sterile" (Fig. 2f). Any fly without observable dye markings should be enclosed in a circle and labeled as "Unmarked" (Fig. 2e). Colour red pens or fluorescent-coloured pens should not be used for labeling to prevent confusion.

2.5 Second step: Observation of flies through epifluorescence microscopy

Flies that were previously circled and labeled as "unmarked" should be transferred to a new gridded sheet separating the head from the body. This new gridded sheet is narrower (6 x 25 cm) than the previous one to leave enough room to manage the sheet under the epifluorescence microscope (Fig 3b). It has two rows of boxes (11 boxes of 2 x 2 cm), the fly's body is placed in the upper row (top of Figure 3a) and the head in the lower row (bottom of Figure 3a).

When observing the fly's head using an epifluorescent microscope (Fig. 3b), focus should be on the ptilinal suture, which has an inverted "U" shape. If the head is not located in the proper position, it should be moved so that the ptilinal suture can be completely visualized through the microscope.

Sufficient observation time should be allocated to detect the fluorescence on the head. Fluorescence is easily observed in the curvature of the inverted "U" (Fig. 3c), where the fluorescent mark is internally consistent. **If the fluorescent mark is observable the fly is marked and therefore should be determined as "sterile".**

If there is the slightest doubt about the marks because they are not observable or are deficient, (few particles are dispersed throughout the head), or there is suspicion of dye contamination (Figs. 3d, 4a-d and Fig. 5a), the fly should undergo an additional process for flies with marking deficiency.

2.6 Third step: Observation of flies having deficient dye marks

The head and body of the flies with deficient dye mark should be washed in a vial with xylene, and then shaken for approximately one minute to eliminate most of the glue that may still be adhered to them. Afterwards, the body is transferred to a saline solution **(Appendix 3)** for 5 to 60 minutes, depending on how preserved the specimen is. During this processes the fly's head and body should be accompanied by a label with the unique trap code, so that the exact head-body match is kept.

Then the fly head is observed in an epifluorescent microscope using the following procedure:

a) On a small piece (3 x 3 cm) of filter paper (Whatman Grade No. 3), place the head on a small drop of clean glue (with no dye contamination) to facilitate its handling. The head's rear region should face the observer so that the occipital orifice can be easily observed (Figure. 5b).

b) Under a simple stereoscopic microscope, open the head via the occiput using extra fine-tipped forceps and then separate the parts of the head sideways to expose the inside of the ptilinal pouch.

c) Check thoroughly with an epifluorescent microscope for the presence of dye marks in the inner folds of the ptilinal pouch (Figure. 5d-f).

If the fly's head presents internal markings, even if these are minimal particles, it should be determined as "sterile fly" (Fig. 5d).

If the fly's head does not present internal markings (Figure. 5f), the fly's reproductive organs should undergo the cytohistological study as described in **Sections 3 and 4** to determine the sterile or fertile condition of the unmarked captured flies.

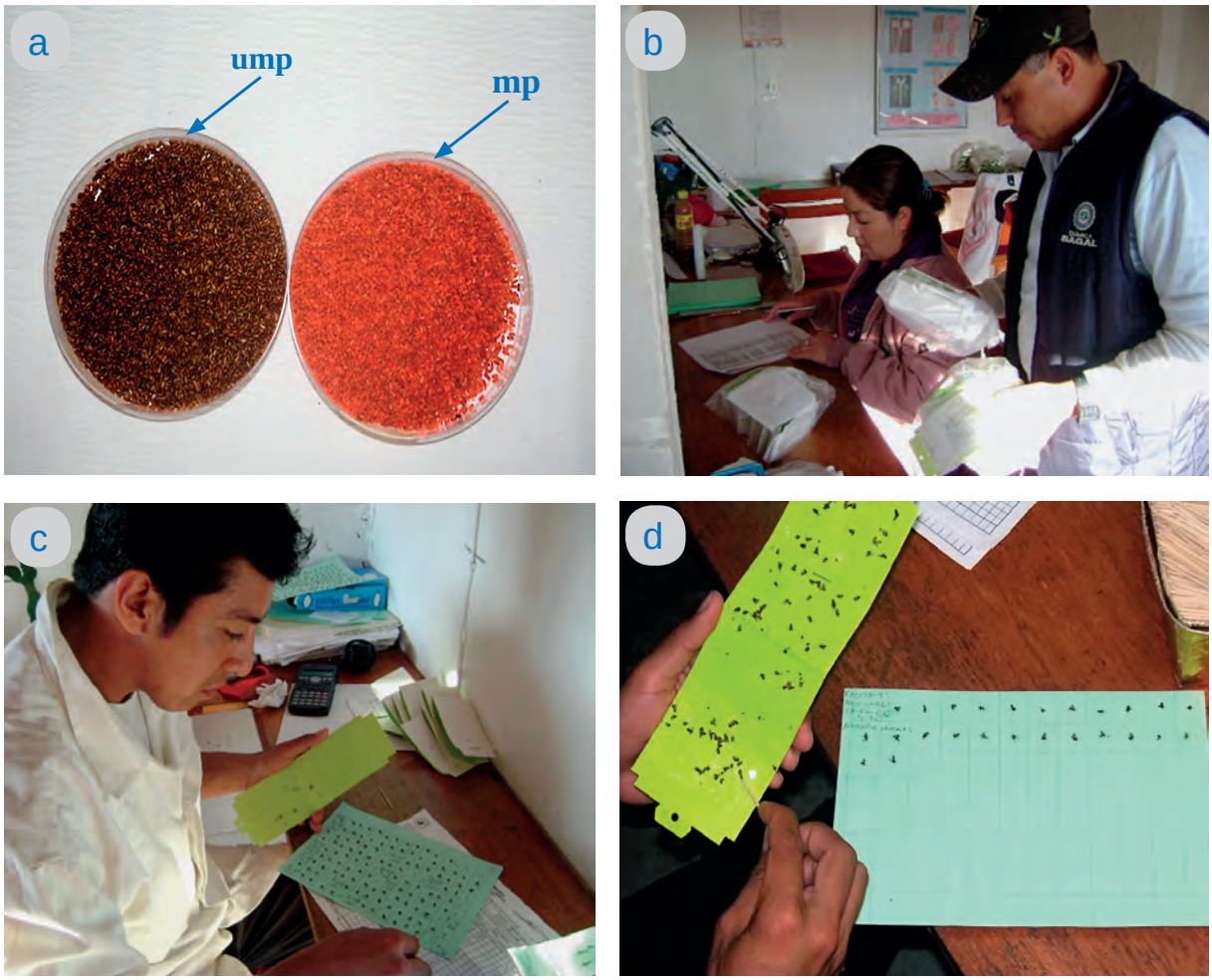

Figure 1. Handling of captured flies: a) Pupae marked with fluorescent dye, ump=unmarked pupae, mp= marked pupae; b) Arrival and revision of trap's sticky inserts in the laboratory; c) and d) Removal of flies from the sticky inserts -green color-; and flies ordered on a gridded sheet.

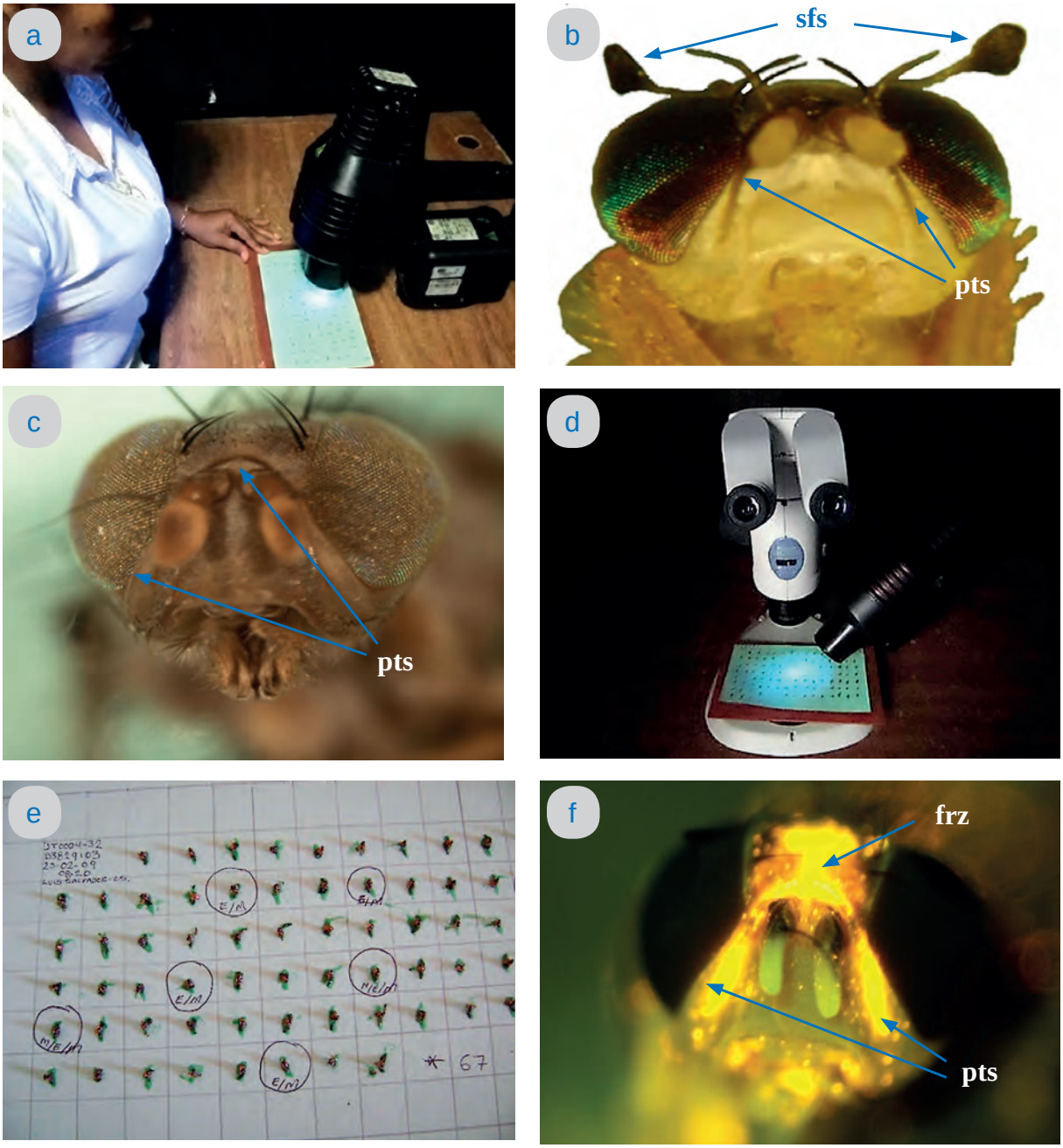

Figure 2. Observation of flies through UV light. a) Direct observation under UV light lamp (Black Ray model B-100AP); b and c) Frontal position for viewing the ptilinal suture; d) Observation under a stereomicroscope and UV light lamp; e) Flies not having dye markings are enclosed in a circle labeled with a code; and f) Ptilinal suture and well-marked frontal zone: sfs = spatulate frontal setae, pts = ptilinal suture, frz = frontal zone.

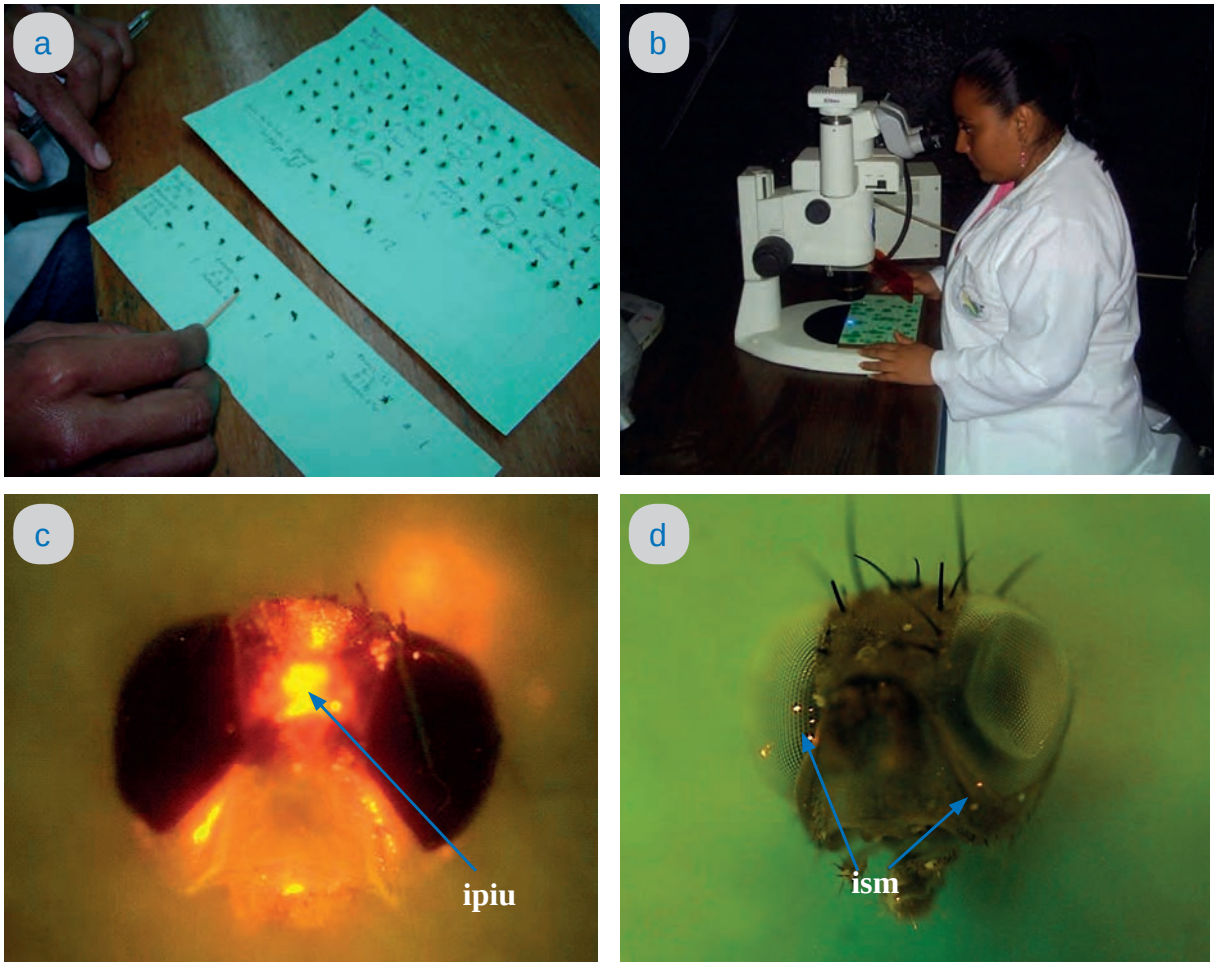

Figure 3. Observation of flies with epifluorescence microscopy. a) Flies with deficient markings on a narrow sheet, at the bottom. Upper row, fly bodies and lower row, fly heads; b) Observation of heads by epifluorescente microscope; c) Head dye marked at the inner part of the ptilinal suture with a shape of inverted "U"; and d) Head with unclear markings. ism= Isolated marks, ipiu= Internal part of inverted "U".

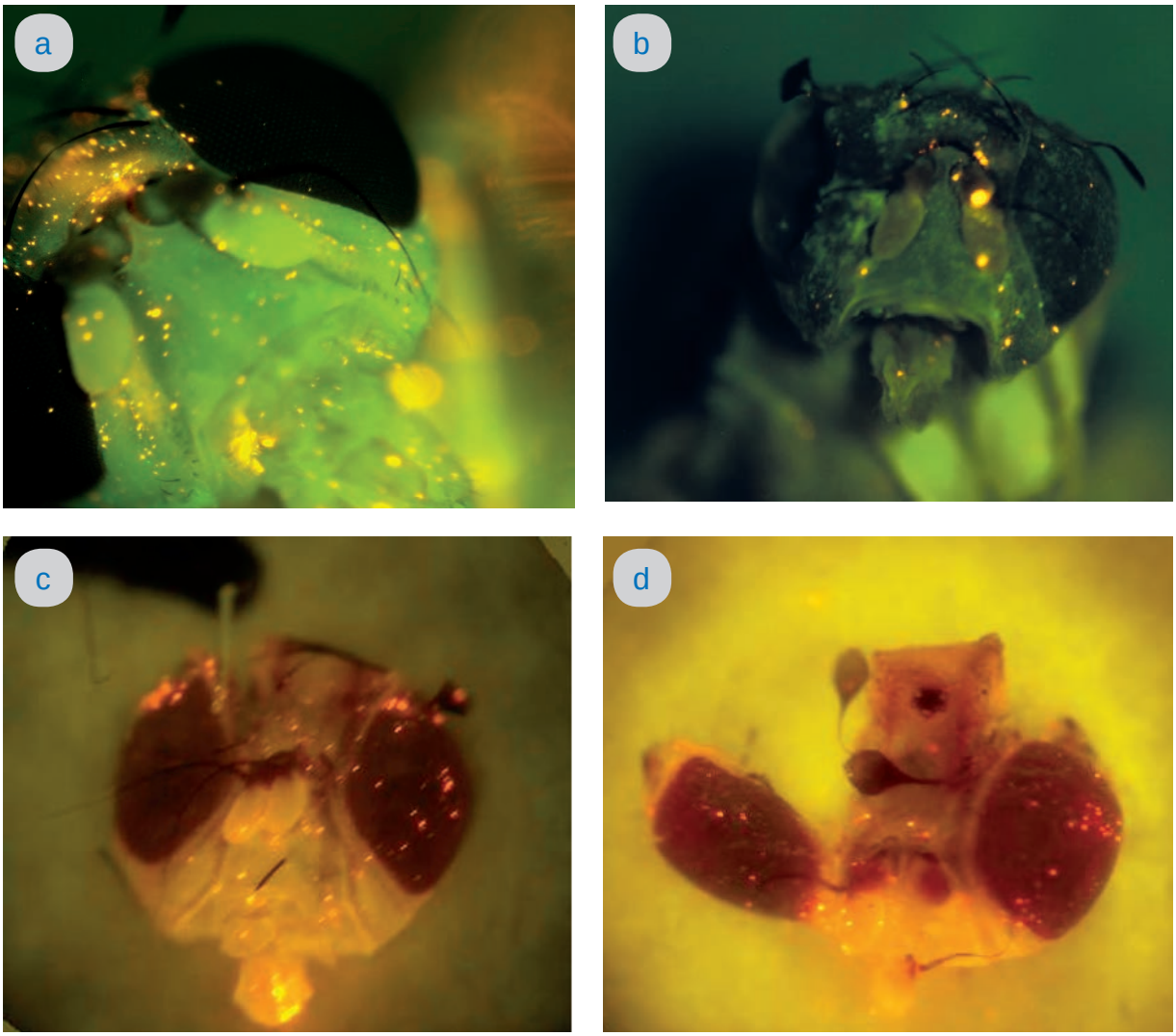

Figure 4. Marking deficiencies. Note the absence of markings on the ptilinal suture in all cases. a) Severe marking contamination at the crescent, base of antennae, facial carina and mouth structures; b) Moderate contamination in the eyes, basal segment of the antenna and genal region; c) Head contaminated in the eyes and frontal zone; and d) Low contamination in the frontal zone and facial carina.

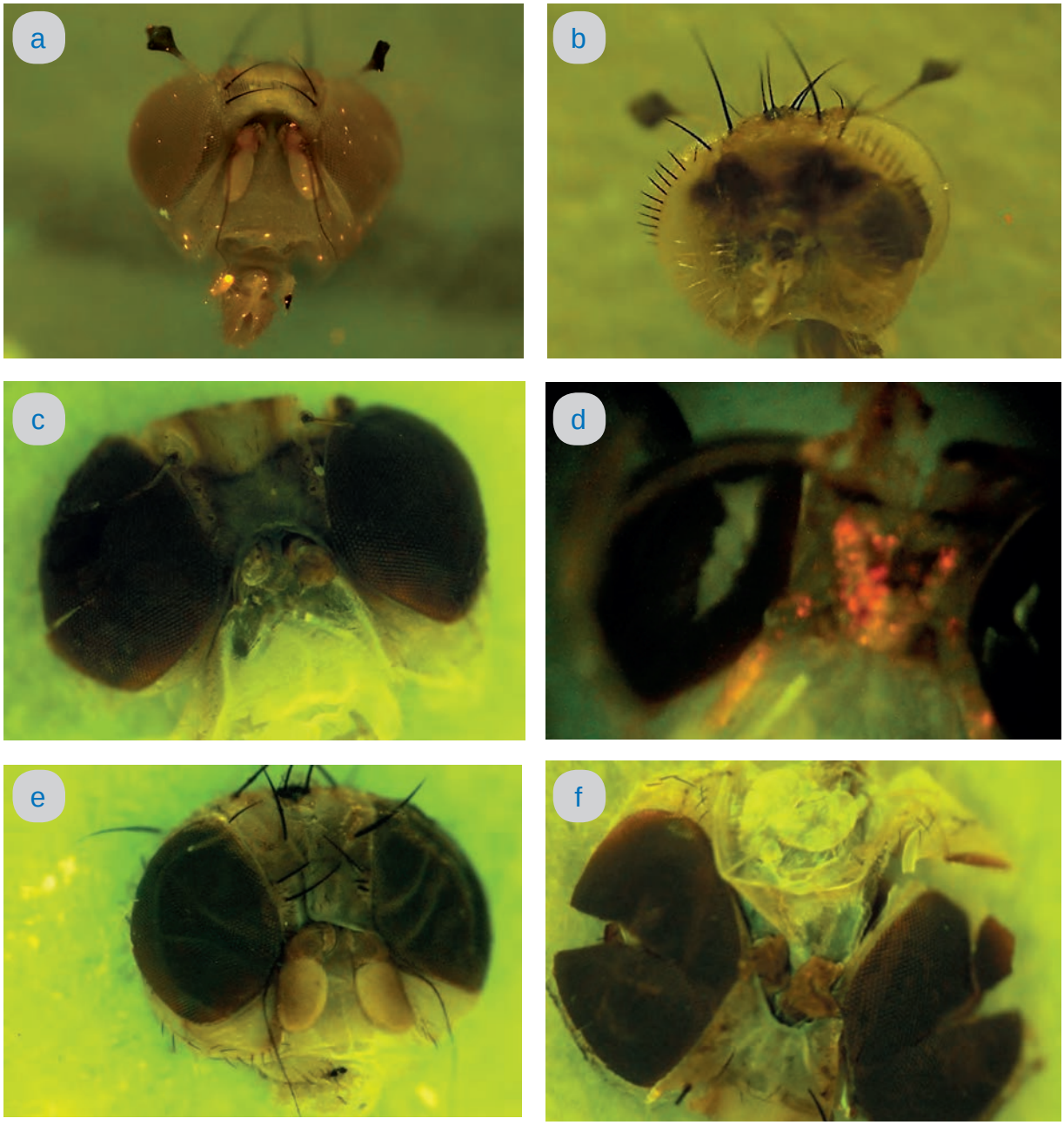

Figure 5. Final determination of the presence/absence of markings. a) Moderate contamination of antennal segments, facial carina and area around the eyes; b) Rear region of the head, exposing the occipital orifice to the observer; c) Head washed with xylene without internal fluorescent markings; d) Internal folds of the ptilinal pouch with residues of fluorescent dye markings of the same head shown in "c"; e) Head washed with xylene without internal fluorescent markings; and f) Internal folds of the ptilinal pouch without residues of fluorescent dye markings of the same head shown in "e".

3. Determination of sterility or fertility **of females from cytohistological** analysis of the ovaries

Even though the genetic sexing system allows for the release of only sterile males, a very small amount of sterile females (1 in 10^3-10^4 flies) may also be released. If unmarked females are captured in the area where Medfly TSL sterile flies are released, these might be either: mature wild females, mature sterile females, immature wild females or immature sterile females. These four cases are discussed in this section.

3.1 Reproductive system of females

The reproductive system of females produces eggs, receives and stores sperm, fertilizes eggs and provides suitable nutrients for hatching and subsequent larval development.

There is substantial confusion in the terminology used to describe the female reproductive system. Different terms or incorrect terminology have been used for the same structures in different tephritidae, mainly of the *Anastrepha* genus. The terminology used here is based on the work of McAlpine (1981) and Norrbom and Kim (1988), and for internal structures particularly on the work of Dean (1935) and Guillen (1983). The reproductive system of a mature Medfly female consists of the following structures (Figures 6 and 7).

Ovaries. A pair of oval-shaped organs of mesodermal origin, somewhat rounded at the anterior end and flattened on the ventral side. They are free in the abdominal cavity, supported by the ovarian tubes that ramify above the thin covering membrane. Each ovary is composed of 20 to 45 polytrophic-type ovarioles; however, in the study conducted to prepare this manual, ovaries with up to 58 ovarioles in mature females older than nine days were found.

Ovarioles. These are the functional units of the ovaries. The germarium, in which the germ cells are contained, is located in the most anterior part of the ovarioles. As soon as the ovarian follicles are formed, each follicle is attached to the preceding and following ones by a row of follicular cells. During vitellogenesis the vitelogenin accumulates in the egg yolk, and then choriogenesis occurs, which is the formation of the chorion or outer covering of the egg. The ovarioles have a thin membrane formed by flat cells that make each ovariole independent. At the anterior extreme, this membrane is extended to form the terminal filament. As the follicles mature, the basal oocyte elongates, and trophocytes grow in the apical part of the follicle, reaching their largest size when the oocyte occupies almost half of the follicle and then degenerate and the follicular cells synthesize the chorion. When the oocyte completes its growth, the follicle cover breaks at the back, and the mature oocyte can then continue through the remaining genital ducts where the follicular cover is reabsorbed.

Lateral oviducts. They are short conical structures of mesodermal origin wide in the area close to the ovary where the pedicels of the ovarioles discharge.

Common oviduct. It is formed by reuniting the two lateral oviducts, it flows into the long vaginal canal, ending in the aculeus. In its anterior part, there is a bulky zone where the bursa copulatrix is most likely located, which is formed by a cuticular extension of the ventral wall called the ventral receptacle. The flow ducts of the spermatheca and the accessory glands end at the dorsal part of the bulking area.

Accessory glands. Translucent, heart-shaped structures occurring in pairs in the lateral and ventral areas of the ovaries, they are connected through the gland ducts to the dorsal part of the oviduct. They produce secretions that are discharged into the glands, where they accumulate. The secretions likely serve among other roles to lubricate the eggs as they descend through the common oviduct.

Spermathecae. Two sclerotised bodies, pear-shaped, black-coloured, where the sperm is stored. These are surrounded by a glandular epithelium coating the secretory epithelium. Fat cells can adhere to the protective cover of the spermathecae, and muscle cells comprise the duct. The latter are most likely responsible for the discharge of a small number of sperm when the micropyle of the oocyte passes through the vagina's expanded region. These organs are connected to the dorsal part of the vagina through the spermathecal ducts.

Aculeus. Also known as ovipositor, it is a highly sclerotised structure connected to the rear section of the vaginal duct. This highly sclerotized structure is inside of the oviscapt and contains the vent through which the eggs come out to be laid.

Oviscapt. It is the elongate, tubular or conical, basal part of the ovipositor, and at the end it presents an eversible membrane. It contains the aculeus.

Signum. Two dark brown sclerotized pieces located in the dorsal wall of the vaginal duct.

Vagina. Elongated genital duct on which the common oviduct, the accessory gland ducts and the spermathecae discharge. It ends in the gonopore located on the 9th abdominal segment.

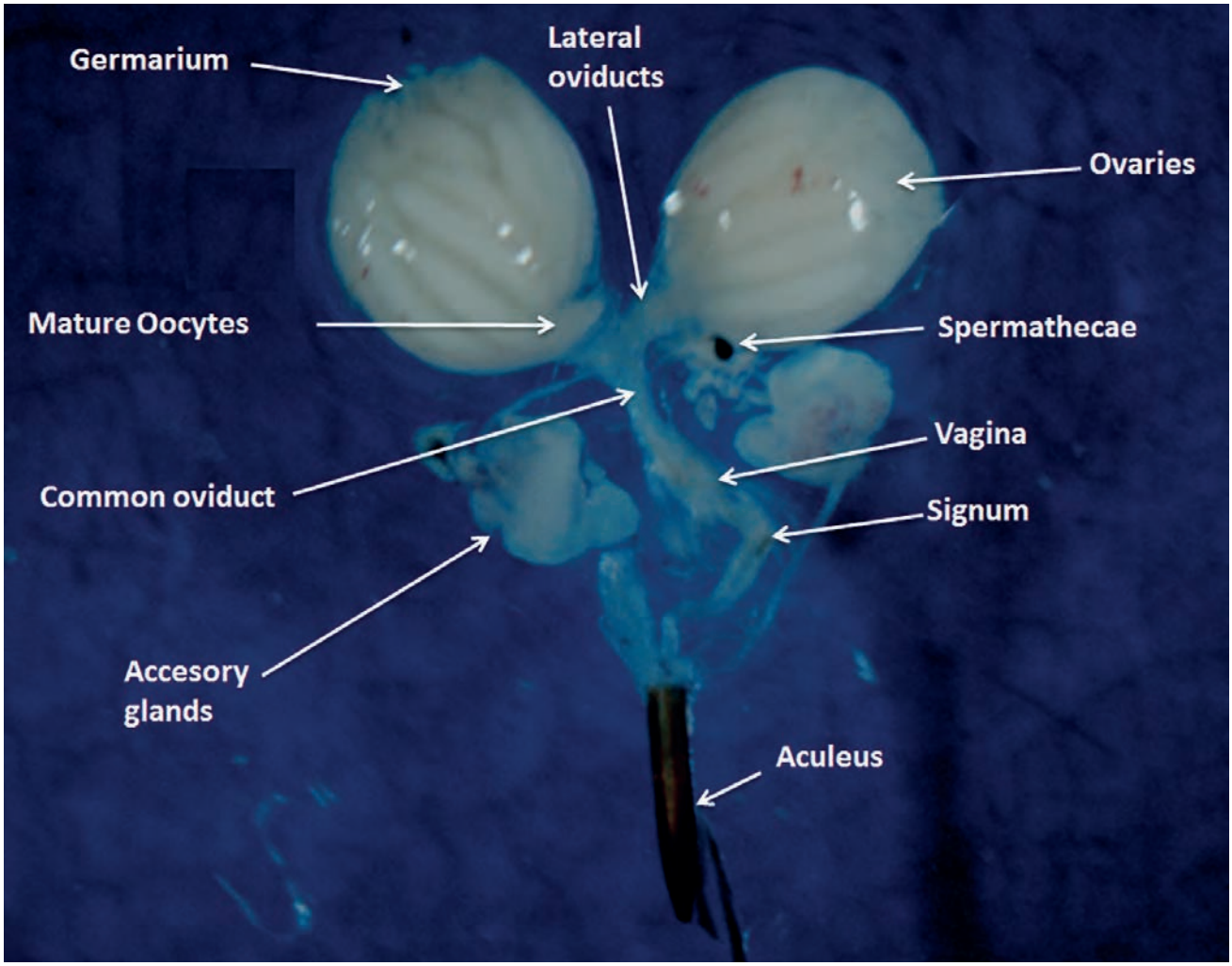

Figure 6. Reproductive system of a wild mature Medfly female at 12 days of age.

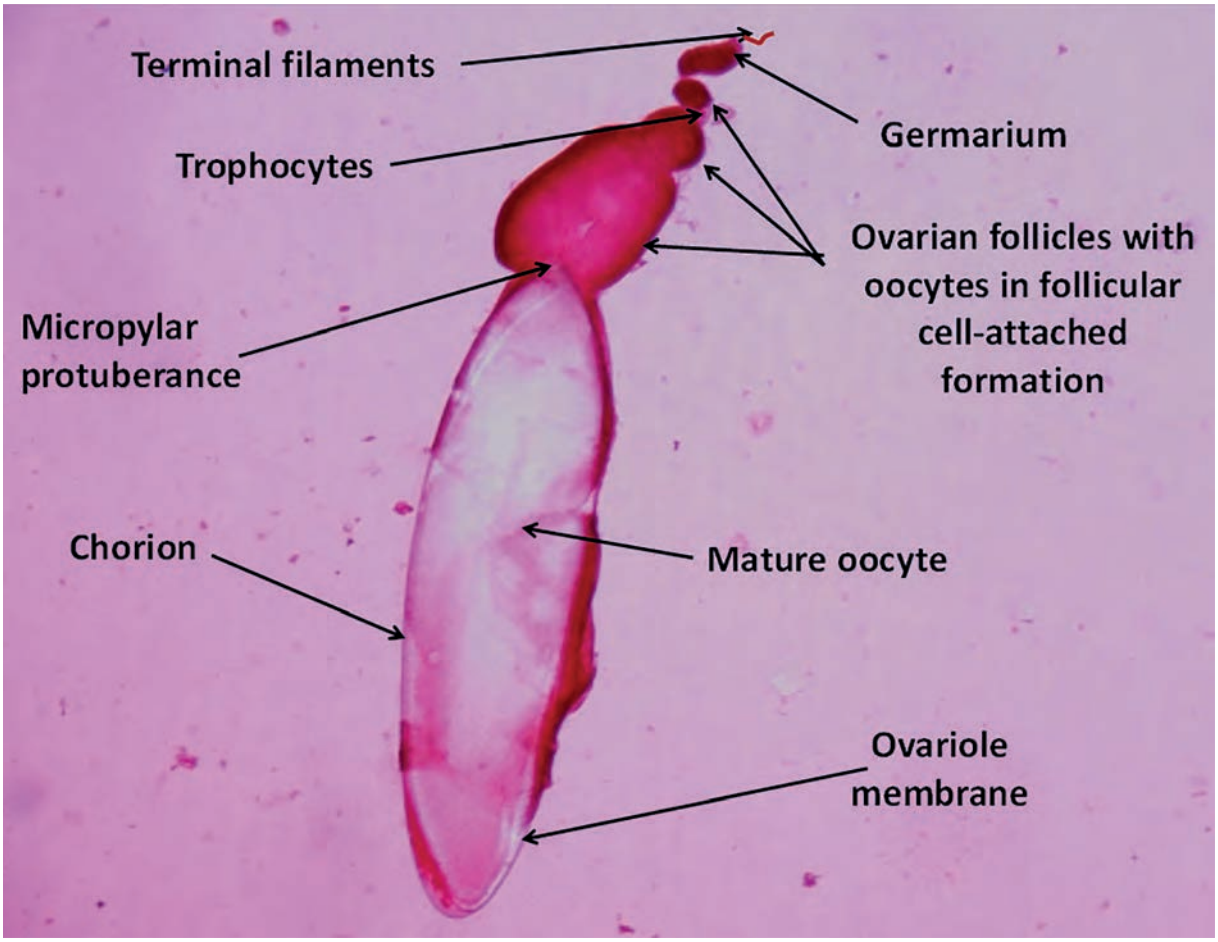

Figure 7. Ovariole from a wild mature Medfly female at 12 days of age stained with aceto-orcein.

3.2 Sexual maturation of females and effect of irradiation on the ovaries

At the time of emergence, fruit fly females are sexually immature. The ovarian maturation process depends on many factors, including temperature, photoperiod, diet, chemical signals and availability of males. Medfly females cannot maintain high fertility without the ingestion of water and essential nutrients for ovarian and egg development, such as carbohydrates, particularly yeast, amino acids, B vitamins and salts. Mass-reared strains usually have shorter maturation periods compared to wild flies because females have previtellogenic follicles from emergence. Mass-reared strains of Medfly also have shorter preoviposition periods of 3-4 days.

Under normal conditions, as sexual maturation develops, wild and non-irradiated TSL females show a clear increase in the size of the ovaries and in the number of mature oocytes, whereas irradiated TSL females show a severe atrophy of the ovaries. These variations are the basis to establish major morphological, morphometric and histological differences between the ovaries of wild, mass-reared non-irradiated females and mass-reared irradiated TSL females.

Flies are irradiated in the late pupal stage, one to two days before adult emergence, but oogenesis is initiated after adult's emergence so the ovaries show no differences in the first days (0 – 2) of fly development; consequently it is not possible to observe cytohistological differences among wild mass-reared non-irradiated and mass-reared irradiated TSL females at that age.

When ovarian growth has begun, on day 2 for the mass-reared non-irradiated TSL females and days 3 - 4 for the wild females, the differences are clear enough to be able to easily discriminate between these two types of females from the irradiated ones. Ovarian growth in the irradiated female flies is not observable; instead, the disintegration of the germarium leaves large empty spaces that can be detected.

The presence of mature oocytes in most ovarioles of the ovaries of non-irradiated TSL females after 2 days of age and after 3 - 4 days of age for wild flies indicates that they are sexually mature; whereas irradiated females continue to lack ovarian growth.

Stable production of mature oocytes is reached between day 7 and 8 in TSL non-irradiated females and wild females, therefore differences between them are undetectable after 7-8 days. Nevertheless they both are easily distinguished from TSL irradiated females since damage to ovaries from irradiation is evident on day 7.

A practical guideline for rapid differentiation among wild, mass-reared non-irradiated TSL and mass-reared irradiated TSL females is the following:

1. Wild females from days 0 - 3, and sometimes up to day 4, show features of being sexually immature.
2. Non-irradiated TSL females from 0 - 2 days shows features of being sexually immature.
3. Irradiated TSL females from 0 - 2 days shows features of being sexually immature, therefore these cannot be clearly distinguished from the wild and non-irradiated TSL females.
4. Wild females present features of sexual maturity from days 4 - 5 up to day 7 or 8.
5. Non-irradiated TSL females present features of sexual maturity from days 3 - 7 and up to day 8.
6. Irradiated TSL females do not present features of sexual maturity, but instead disintegration of the germarium leaves large empty spaces in the ovaries.
7. Wild females present full sexual maturation at days 7 or 8.
8. Non-irradiated TSL females present full sexual maturation at days 7 or 8.
9. Irradiated TSL females present clear and evident irradiation damage on day 7.

However, for a more complete description of ovary features and irradiation damage to diagnose the sterility or fertility of females it is recommended to go to **Section 3.5**.

3.3 Determination of the sterile or fertile condition of females

Dissection of ovaries

The female abdomen is placed in a vial with saline solution **(Section 2.6).** The abdomen is then placed on a microscope slide in a dorsal position so that the ovipositor points towards the observer. Using magnification lenses (10x to 30x) of a stereomicroscope and extra fine-tipped entomological tweezers No. 5, the dorsal and ventral abdominal segments are carefully separated. Once the interior of the abdomen is exposed, the female's reproductive system is dissected very delicately to avoid rupturing its different structures (ovaries, spermatheca, accessory glands, etc.). The dissection should be performed with the abdomen immersed in distilled water to avoid the dehydration of the structures. In order to focus on the observation of the ovaries, any remaining tissues that are not of interest to the study should be carefully removed to avoid rupturing any of the structures.

Sterility or fertility of mature females

If the female is mature (3 days or older), it is easy to determine whether it is fertile or sterile by studying its ovaries. When the ovaries are at an advanced stage of maturity, they are quite bulky and take up most of the fly central abdominal region. Ovaries with one or more mature oocytes are fertile; therefore the **females bearing ovaries with mature oocytes should be determined as fertile** (Fig. 6).

When the ovaries are immature or atrophied by irradiation, they are located in the central abdominal region at the ends of each lateral oviduct. The ovaries seem to be destroyed, or under development, or with not well

defined ovarioles. They should be carefully observed under the compound microscope to determine their sterile or fertile condition. Therefore, these ovaries are transferred to another microscope slide, stained with a drop of aceto-orcein and covered with a slip for observation (**Appendix 4**). If a permanent mounting is desired, dissected ovaries and adjacent structures should be transferred to an excavated slide and covered with mounting medium (Canadian balsam, Hoyer liquid, or Entellan). If the microscopy equipment has a digital camera installed on it, it is worthwhile to take microphotographs at different magnifications to have a long-lasting record of the mounting.

Ovaries that present clear severe atrophy, with large empty spaces due to the irradiation damage are sterile; therefore **females bearing damaged ovaries should be determined as sterile** (Figure 15 c and i).

In this latter case, if there are doubts about the maturity of the ovaries, an additional element is the observation of the spermatheca to determine if the female had successfully mated or not.

Observation of the spermathecae. These are located below the ovaries and very close to the accessory glands. Both spermathecae should be separated from the original mounting and placed immediately on another microscope slide with a drop of distilled water. The dissection should be performed very carefully to avoid the breakage and dehydration of the organs.

Once the spermathecae are in the desired position and free from water and other tissues, a drop of aceto-orcein dye should be added. 3 – 4 minutes are required to get the organs stained. To expose the content of the spermathecae, a cover slip is placed over the spermathecae and firmly squeezed until they crush.

Observing their content through a compound microscope at magnifications between 45x and 100x, the sperm shows a filiform aspect, and it is also entangled in the same way when it is located at the base of the testes of mature males. Otherwise, only glandular fluid or epithelial cells without the presence of sperm will be observed (Figure16d-f).

Presence of sperm in the spermatheca is a clear reconfirmation that the female was mature and had mated.

Sterility or fertility of immature females

As it is described in **Section 3.2**, it is not possible to observe cytohistological differences among wild and irradiated females of 0 – 2 days of age, so clear determination of the sterile or fertile condition of immature females is not possible. Although there is a risk of capturing unmarked immature sterile TSL females, the probability of such event occurring is highly unlikely since:

- Sterile flies are released in the field once sexual maturation has begun (4 days old).
- Sterile flies are marked before they are released, efficiency of marking is above 99%; therefore the number of unmarked flies released is very low.
- Percentage of TSL females released in the field together with TSL males is less than 0.1%. Consequently the number of sterile females present in the field that could be captured is quite low.
- Percentage of sterile females recaptured by the trapping network is even lower in food-baited traps in view that sterile females have been fed during maturation.

For instance, the Programa MOSCAMED in Mexico, where sterile TSL flies are continuously released along the border with Guatemala has recorded that from January 2009 to April 2010, 456,650 sterile male flies together with only 282 sterile females were recaptured. The females recaptured represented only 0.06% of the total recaptured flies. In addition, all males and females were marked and not a single immature sterile female was captured. Furthermore, records of several years show that 3,860 sterile females have been captured and only three females were unmarked, being all of them mature.

Even though determination of sterility or fertility of females can easily be done following the steps described in **Section 3.3**, if doubts still remain, the final determination of the sterile or fertile condition of females can be made based on the technical criteria described in Section 3.4.

3.4 Ovarian maturation in wild and non-irradiated VIENNA Medfly TSL females compared with VIENNA Medfly TSL females irradiated one day before emergence with 100, 125 and 145 Gy

Day 0 (Emergence). (Fig 8a-f, 9, 10 and Tables 1, 2).

Upon emergence, ovaries of irradiated and non-irradiated females are rounded, slightly more long than wide and contain abundant adipose tissue attached to the outside and tracheal ramifications that extend inward to provide oxygen to the organs. The ovarian cover is thin but already defined. The germaria are already present, are larger than in mature females and occupy much of the anterior ovary. In each ovariole can be observed from one to up to three round-shaped ovarian follicles, as vitellogenesis has already started. Although these follicles are less intensively stained with aceto-orcein in irradiated flies than in non-irradiated flies, it is not possible to establish consistent histological differences. The average sizes of ovaries range from 0.383 to 0.395 mm long by 0.260 to 0.267 mm wide, with no significant differences between groups.

Day 1. (Fig. 8g-i, 9, 10, 11a-c and Tables 1, 2).

The average ovary length of the TSL females irradiated with 100 Gy increases slightly to 0.413 mm, but there are no significant differences among the groups. The other features remain similar as on day 0. The ovarian follicles in the irradiated flies are less stained; however, it is not possible to establish solid differences with the non-irradiated groups.

Day 2. (Fig. 9, 10, 11d-i and Tables 1, 2).

The ovaries of non-irradiated wild and TSL flies begin to rapidly grow on day 2, with the sizes in the TSL group reaching 0.614 x 0.428 mm. In these ovaries, the amount of adipose tissue is reduced, and the ovarioles, most of which contain four ovarian follicles, have grown considerably. Basal oocytes are very elongated and visible without aceto-orcein staining. It is possible that choriogenesis is initiated during this stage. The differentiation between the ovaries of the non-irradiated TSL flies from those of the wild and irradiated flies is possible both morphometrically and histologically. Although the ovaries of the wild flies reach an average size of 0.435 x 0.315 mm, it is not yet possible to differentiate elongated basal oocytes, and they are not visible without aceto-orcein staining.

The ovaries of the three irradiated groups (100, 125 or 145 Gy) still have nodules of adipose tissue, tracheal tubes and few, lightly stained ovarian follicles that cannot be clearly differentiated because they are similar in average size (0.383 x 0.258 mm). These histological features make it difficult to definitively differentiate these ovaries at this stage from those of the wild flies.

Day 3. (Fig. 9, 10, 12, 13a-f and Tables 1, 2, 3).

Remarkable growth is observed in the ovaries of non-irradiated TSL flies, which acquire ovoid shapes and reach average sizes of 0.991 x 0.657 mm, which is 2.5 times larger than on the day of emergence. Their ovarioles contain the greatest number of ovarian follicles with basal oocytes, which have also grown considerably and can be observed without aceto-orcin staining by a stereomicroscope with 25x magnification. Follicular covers rupture, and mature oocytes moving through the follicular ducts may be the first to be oviposited, as observed in females during advanced development in which mature oocytes can be detected descending into the lateral oviducts. In these individuals, two to ten mature oocytes can be found per ovary. The ovaries of wild flies also increase in size and have similar characteristics to those of non-irradiated TSL flies at two days of age; however, in some specimens, oocytes that mature early can be detected. In females during early development, elongated basal oocytes with well-defined germinal zones are observed when aceto-orcein is used to stain the ovaries.

The ovaries of the three groups of irradiated flies are slightly increased in size, with rounded ovaries slightly more long than wide with an average size of 0.379 x 0.255 mm and, similar to the previous day, adipose tissue and tracheal tubes. It is possible to observe that the germarium area is less stained, likely due

to the disintegration of cells in the germaria. Ovarian follicles have rounded shapes and are stained with less intensity than in the non-irradiated groups, and evidence of morphological and histological differences is observed between both groups.

Day 4. (Fig. 9, 10, 12, 13g-i, 14a-c and Tables 1, 2, 3).

Ovarian development continues, and the ovaries of non-irradiated TSL flies measure on average 1.204 ± 0.163 mm long and 0.876 ± 0.154 mm wide. They are ovoid in form, and most have very elongated ovarioles with basal oocytes that occupy almost half of the lower ovary and are visible to the naked eye or with a stereoscope at 25x magnification. The germaria have decreased in size compared to the first few days and are oriented at random in the front; together with the terminal filaments, they form a network that surrounds the ovary at its apical part, giving it a rounded shape. At this age, between 2 and 28 (an average of 10.06 ± 7.52) mature oocytes can be found in the ovaries of most females, indicating that not all females have reached the same stage of maturity. The ovaries of wild females also continue to grow in size, reaching an average length of 0.623 ± 0.083 mm and average width of 0.427 ± 0.112 mm, with similar characteristics to the non-irradiated TSL flies at days 2-3; however, it is possible to find ovaries with up to 11 or 12 mature oocytes and with large elongated basal oocytes in full choriogenesis. These oocytes are visible to the naked eye or with a stereoscope at a 25x magnification.

Ovaries of the three irradiated groups grow minimally and maintain an average length of 0.402 mm and width of 0.270 mm with no significant differences between these groups (Tables 1 and 2). In all irradiated groups, adipose tissue continues to adhere to ovary surfaces, and there are also tracheal branches around the organs. Staining with aceto-orcein reveals a decrease in the number of cells in the germarium area and round ovarian follicles, some of which are developing; some start disintegrating, leaving large intercellular spaces, and the remaining stain with less intensity than in previous days.

Days 5, 6 and 7. (Fig. 9, 10, 12, 14d-i and Tables 1, 2, 3).

Over days 6-7, the increase in ovary size continues both in the non-irradiated TSL flies and in wild flies. The difference between these groups decreases until day 7, with an average size of 1.245 x 1.013 mm in the TSL flies and 1.189 x 0.907 mm in the wild flies. The production of mature oocytes is widespread, and the ovarioles of all non-irradiated TSL females have mature oocytes ready to be oviposited, described as “the egg load” by some authors (Kendra et al., 2006). However, a wide variation is found in the number of mature oocytes per ovariole, ranging from 5 to 26 with averages of 14.4, 11.2 and 12 on days 5, 6 and 7 respectively, which indicates that there are clear differences in ovarian development even within groups of the same age and in their egg-laying ability, which can be due to the early sexual maturity of these females. This variation also applies to the wild females, despite a lower egg load observed with respect to the TSL flies and with a wider variation, as the number of mature oocytes in the ovarioles range from 0 to 32, with averages of 8, 9 and 9.2 on days 5, 6 and 7 respectively. These averages are lower because the quantities are minimal in some females, indicating a greater difference in sexual maturity among the wild females than in the non-irradiated TSL females.

In irradiated TSL flies during this stage, ovaries can still lengthen and become slightly wider, culminating on day 7 with sizes between 0.502 x 0.270 mm and 0.518 x 0.276 mm, with no significant morphometric differences between the groups. The adipose tissue and tracheal ramifications are very fibrous on the external areas of the ovaries of the irradiated TSL flies. By staining these structures with aceto-orcein, fewer germarium cells are weakly stained, and it is still possible to detect rounded ovarian follicles that slowly disintegrate leaving intercellular spaces; the remaining cells are stained with less intensity than on the previous days. However, establishing clear histological differences among the irradiated TSL flies on different days is still risky.

Days 8 to 20. (Fig. 9, 10, 11a-c, 15a-f and Tables 1, 2, 3).

This age range is characterized by stable morphometric, histological and physiological parameters in all groups. At this age, the ovary size of non-irradiated TSL and wild flies are maintained in the range of 1.201 ±

0.204 to 1.353 ± 0.079 mm long and 1.047 ± 0.016 to 1.149 ± 0.100 mm wide without significant differences between the two groups. The fluctuations between the two groups and within the same group are most likely due to interphases of oviposition and pauses. Some individuals may be found with ovary sizes of up to 1.750 x 1.450 mm, with atrophied lateral oviducts that hamper oviposition and most likely cause the large volume of the ovaries. In general, the ovaries are ovoid-shaped and slightly flattened at the apical region, although in some instances they are rounded or globular, and the covering membrane is thinner and translucent. The ovarioles are elongated and visible to the naked eye or with minimal magnification in a stereoscope and have ovarian follicles at different stages of development, and basal follicles with numerous off-white mature oocytes ready to be oviposited can be observed descending the lateral oviducts. The spermathecae can be observed with surrounding adipose tissue, and accessory glands are translucent and full of secretions. Based on these features, we can consider that these females are at an advanced stage of sexual maturity because the ovaries are in full egg production. Although there is variation in the egg load among flies of the same age, the average egg load fluctuates between 14.2 ± 5.9 and 28.3 ± 10.6 mature oocytes per ovary in the irradiated TSL flies and between 12 ± 3.6 and 26.3 ± 8.9 per ovary in wild flies, with both groups reaching maximum values between 12 and 19 days of age (Table 3). It is possible to detect ovaries with record numbers of total mature oocytes, with 57 and 60 in non-irradiated TSL flies at day 15 and 48 in wild flies on day 17.

The ovaries of the three irradiated groups of females have stable growth, and the ovaries range in size from 0.482 to 0.526 mm long and 0.258 to 0.325 mm wide, without statistically significant morphometric differences among these groups (Tables 1 and 2). Histologically, the ovaries grow but less adipose tissue is observed because fat is used as an energy source during the process of oogenesis. At this point, the process is inhibited, the thin membrane that covers the ovaries has a fibrous aspect, and the branched tracheal tubes present in both ovaries are stiffer than in previous days and acquire irregular shapes. Accumulating adipose tissue can also be detected around the spermathecae. The accessory glands are observed to be turgid due to the secretions produced. During the mounting of these irradiated ovaries with apical aceto-orcein staining, it is difficult to detect germaria, and many empty spaces are observed as the cells disintegrate. In a few cases, remains of developing follicles are found, but they are scarce, hardly stained and rounded. With the confirmation of these features by careful and meticulous histological analysis, as long as clean and complete mountings are made of these ovaries, it is possible to diagnose them as sterile flies with advanced maturity using these chronological age ranges.

Using all the elements described above, wild and non-irradiated TSL flies can be easily separated from the irradiated flies by the advanced stage of ovarian maturity reached in the first group, compared to the completely ceased development of the oogenesis in the irradiated females.

Day 25. (Fig. 9, 10, 12, 15g-i, 16a-c and Tables 1, 2, 3).

At this age, no significant changes are detected in the non-irradiated flies from 8-20 days, and the ovary size in non-irradiated TSL flies remains similar. The ovaries of wild flies can reach their maximum dimensions of length and width at this age, ranging from 1.341 mm ± 0.08 and 1.140 mm ± 0.10, respectively, with large variations within the same group most likely determined by the interphases of oviposition and pauses; however, there are no significant differences with respect to the non-irradiated TSL flies.

The production of mature oocytes and general histological features are very similar to earlier ages, and mature oocyte production continues in all ovarioles from both groups, with a mean of 20.7 ± 9.7 mature oocytes per ovariole in non-irradiated TSL flies and 12.3 ± 2.7 in the wild flies; however, ovaries with up to 35 to 39 oocytes can be found in the TSL flies, and ovaries with 17 to 20 oocytes can be found in the wild flies, which indicates that they remain in a stage of advanced maturity and are producing eggs that are ready to be oviposited.

In all three groups of irradiated flies, both the sizes and the histological characteristics observed at 8 to 20 days are maintained up to 25 days without significant differences among the groups, making it easy to distinguish them from non-irradiated flies of the same ages.

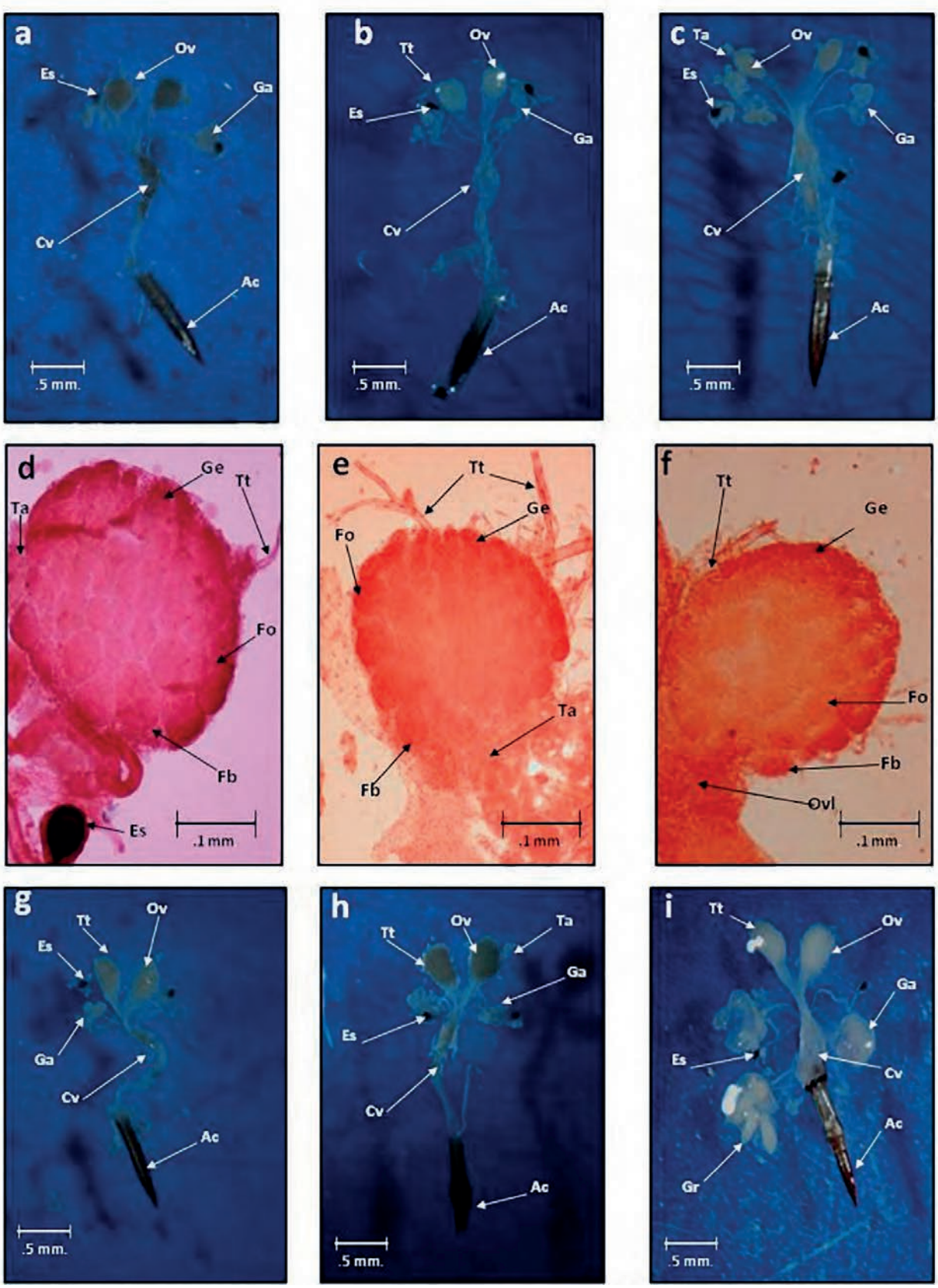

Figure 8. a) Non-irradiated TSL flies at day 0; b) Wild flies at day 0; c) TSL flies irradiated with 100 Gy at day 0; d) Non-irradiated, stained TSL flies at day 0; e) Stained wild flies at day 0; f) Stained TSL flies irradiated with 125 Gy at day 0; g) Non-irradiated TSL files at day 1; h) Wild flies at day 1; and i) TSL flies irradiated with 145 Gy at day 1: Ac= Aculeo, Cv= Vaginal duct, Es= Spermathecae, Fb= Basal follicle, Fo= Ovarian follicle, Ga= Accessory gland, Ge= Germarium, Gr= Rectal gland, Ov= Ovary, Ovl= Lateral oviduct, Ta= Adipose tissue , Tt= Tracheal tube.

Table 1. Ovarian length of non-irradiated TSL flies and of wild and TSL flies irradiated with 100, 125 or 145 Gy (mean ± SD). For each treatment, n = 15 females per day

	Ovarian length (mm)									
AGE (DAYS)	NON-IRRADIATED TSL		WILD		TSL 100 Gy		TSL 125 Gy		TSL 145 Gy	
0	0.386 ± 0.044	a	0.384 ± 0.036	a	0.395 ± 0.036	a	0.385 ± 0.024	a	0.383 ± 0.037	a
1	0.401 ± 0.039	a	0.401 ± 0.037	a	0.413 ± 0.043	a	0.405 ± 0.048	a	0.406 ± 0.032	a
2	0.614 ± 0.088	a	0.435 ± 0.076	b	0.390 ± 0.024	bc	0.375 ± 0.047	c	0.386 ± 0.055	c
3	0.991 ± 0.159	a	0.475 ± 0.064	b	0.379 ± 0.029	c	0.375 ± 0.036	c	0.385 ± 0.046	c
4	1.204 ± 0.163	a	0.623 ± 0.083	b	0.394 ± 0.051	c	0.413 ± 0.049	c	0.399 ± 0.057	c
5	1.255 ± 0.153	a	1.045 ± 0.191	b	0.430 ± 0.038	c	0.427 ± 0.068	c	0.423 ± 0.047	c
6	1.260 ± 0.175	a	1.070 ± 0.188	b	0.485 ± 0.063	c	0.499 ± 0.070	c	0.491 ± 0.048	c
7	1.245 ± 0.103	a	1.189 ± 0.139	a	0.517 ± 0.051	b	0.518 ± 0.054	b	0.502 ± 0.047	b
8	1.291 ± 0.058	a	1.284 ± 0.126	a	0.496 ± 0.043	b	0.517 ± 0.050	b	0.500 ± 0.041	b
9	1.301 ± 0.140	a	1.311 ± 0.146	a	0.501 ± 0.057	b	0.502 ± 0.062	b	0.513 ± 0.056	b
10	1.305 ± 0.123	a	1.311 ± 0.118	a	0.491 ± 0.034	b	0.491 ± 0.047	b	0.503 ± 0.049	b
11	1.353 ± 0.079	a	1.299 ± 0.150	a	0.484 ± 0.052	b	0.510 ± 0.057	b	0.482 ± 0.052	b
12	1.261 ± 0.150	a	1.339 ± 0.161	a	0.491 ± 0.053	b	0.493 ± 0.053	b	0.519 ± 0.059	b
13	1.264 ± 0.160	a	1.201 ± 0.204	a	0.501 ± 0.054	b	0.499 ± 0.042	b	0.483 ± 0.044	b
14	1.268 ± 0.181	a	1.261 ± 0.125	a	0.497 ± 0.044	b	0.493 ± 0.045	b	0.505 ± 0.049	b
15	1.292 ± 0.154	a	1.237 ± 0.138	a	0.493 ± 0.060	b	0.507 ± 0.047	b	0.494 ± 0.058	b
16	1.304 ± 0.181	a	1.304 ± 0.206	a	0.490 ± 0.065	b	0.503 ± 0.061	b	0.495 ± 0.049	b
17	1.328 ± 0.078	a	1.277 ± 0.165	a	0.523 ± 0.052	b	0.511 ± 0.038	b	0.505 ± 0.060	b
18	1.349 ± 0.103	a	1.298 ± 0.163	a	0.497 ± 0.046	b	0.515 ± 0.056	b	0.517 ± 0.065	b
19	1.294 ± 0.110	a	1.327 ± 0.178	a	0.494 ± 0.057	b	0.526 ± 0.052	b	0.494 ± 0.051	b
20	1.311 ± 0.102	a	1.292 ± 0.115	a	0.526 ± 0.060	b	0.503 ± 0.069	b	0.511 ± 0.044	b
25	1.327 ± 0.095	a	1.341 ± 0.080	a	0.519 ± 0.062	b	0.495 ± 0.057	b	0.505 ± 0.043	b

For each value, followed by the same letter in the same row means they are not statistically different (Fisher's exact test, P <0.05).

Table 2. Ovary width of non-irradiated TSL flies and wild and TSL flies irradiated with 100, 125 or 145 Gy. (mean ± SD). For each treatment, n = 15 females per day.

	Ovarian width (mm)									
AGE (DAYS)	NON IRRADIATED TSL		WILD		TSL 100 Gy		TSL 125 Gy		TSL 145 Gy	
0	0.262 ± 0.036	a	0.267 ± 0.031	a	0.265 ± 0.031	a	0.260 ± 0.031	a	0.261 ± 0.038	a
1	0.277 ± 0.038	a	0.277 ± 0.050	a	0.268 ± 0.054	a	0.266 ± 0.040	a	0.264 ± 0.034	a
2	0.428 ± 0.071	a	0.315 ± 0.070	b	0.259 ± 0.026	c	0.260 ± 0.042	c	0.255 ± 0.049	c
3	0.657 ± 0.174	a	0.330 ± 0.072	b	0.259 ± 0.026	c	0.257 ± 0.038	c	0.249 ± 0.023	c
4	0.876 ± 0.154	a	0.427 ± 0.112	b	0.262 ± 0.040	c	0.272 ± 0.034	c	0.278 ± 0.043	c
5	0.913 ± 0.100	a	0.749 ± 0.127	b	0.277 ± 0.041	c	0.272 ± 0.034	c	0.264 ± 0.025	c
6	0.969 ± 0.114	a	0.793 ± 0.110	b	0.297 ± 0.042	c	0.279 ± 0.048	c	0.265 ± 0.035	c
7	1.013 ± 0.104	a	0.907 ± 0.147	b	0.270 ± 0.028	c	0.276 ± 0.043	c	0.274 ± 0.026	c
8	1.075 ± 0.104	a	1.072 ± 0.164	a	0.276 ± 0.030	b	0.262 ± 0.041	b	0.276 ± 0.036	b
9	1.111 ± 0.118	a	1.110 ± 0.112	a	0.280 ± 0.032	b	0.293 ± 0.028	b	0.279 ± 0.030	b
10	1.111 ± 0.104	a	1.108 ± 0.101	a	0.281 ± 0.035	b	0.295 ± 0.036	b	0.289 ± 0.038	b
11	1.147 ± 0.084	a	1.112 ± 0.125	a	0.279 ± 0.031	bc	0.313 ± 0.037	b	0.258 ± 0.020	c
12	1.072 ± 0.107	a	1.083 ± 0.107	a	0.284 ± 0.031	b	0.297 ± 0.045	b	0.277 ± 0.042	b
13	1.083 ± 0.114	a	1.077 ± 0.086	a	0.297 ± 0.057	b	0.283 ± 0.025	b	0.281 ± 0.039	b
14	1.066 ± 0.116	a	1.047 ± 0.116	a	0.299 ± 0.046	b	0.317 ± 0.042	b	0.297 ± 0.047	b
15	1.082 ± 0.121	a	1.053 ± 0.102	a	0.310 ± 0.055	b	0.293 ± 0.066	b	0.307 ± 0.060	b
16	1.127 ± 0.145	a	1.126 ± 0.118	a	0.288 ± 0.031	b	0.300 ± 0.039	b	0.285 ± 0.031	b
17	1.149 ± 0.100	a	1.119 ± 0.120	a	0.313 ± 0.046	b	0.310 ± 0.023	b	0.306 ± 0.038	b
18	1.110 ± 0.107	a	1.102 ± 0.102	a	0.289 ± 0.066	b	0.290 ± 0.029	b	0.293 ± 0.058	b
19	1.111 ± 0.114	a	1.134 ± 0.140	a	0.281 ± 0.038	b	0.320 ± 0.042	b	0.284 ± 0.050	b
20	1.133 ± 0.121	a	1.110 ± 0.103	a	0.325 ± 0.051	b	0.306 ± 0.055	b	0.307 ± 0.055	b
25	1.123 ± 0.092	a	1.140 ± 0.100	a	0.332 ± 0.042	b	0.309 ± 0.051	b	0.310 ± 0.033	b

For each variable, followed by the same letter in the same row means they are not statistically different (Fisher's exact test, P < 0.05).

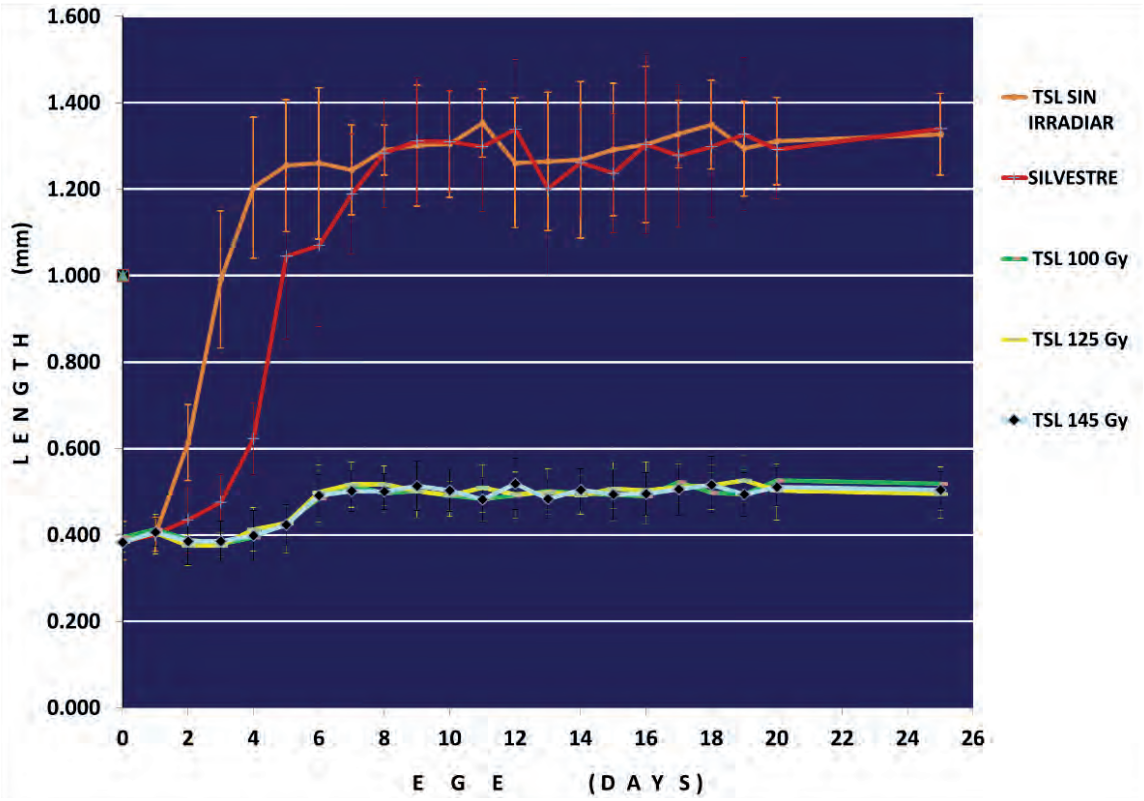

Figure 9. Ovary length of *C. capitata* undergoing the following treatments: non-irradiated TSL flies and wild and TSL flies irradiated with 100, 125 or 145 Gy from emergence (day 0) to 25 days of age. Vertical bars represent the standard error. For each age, n = 15

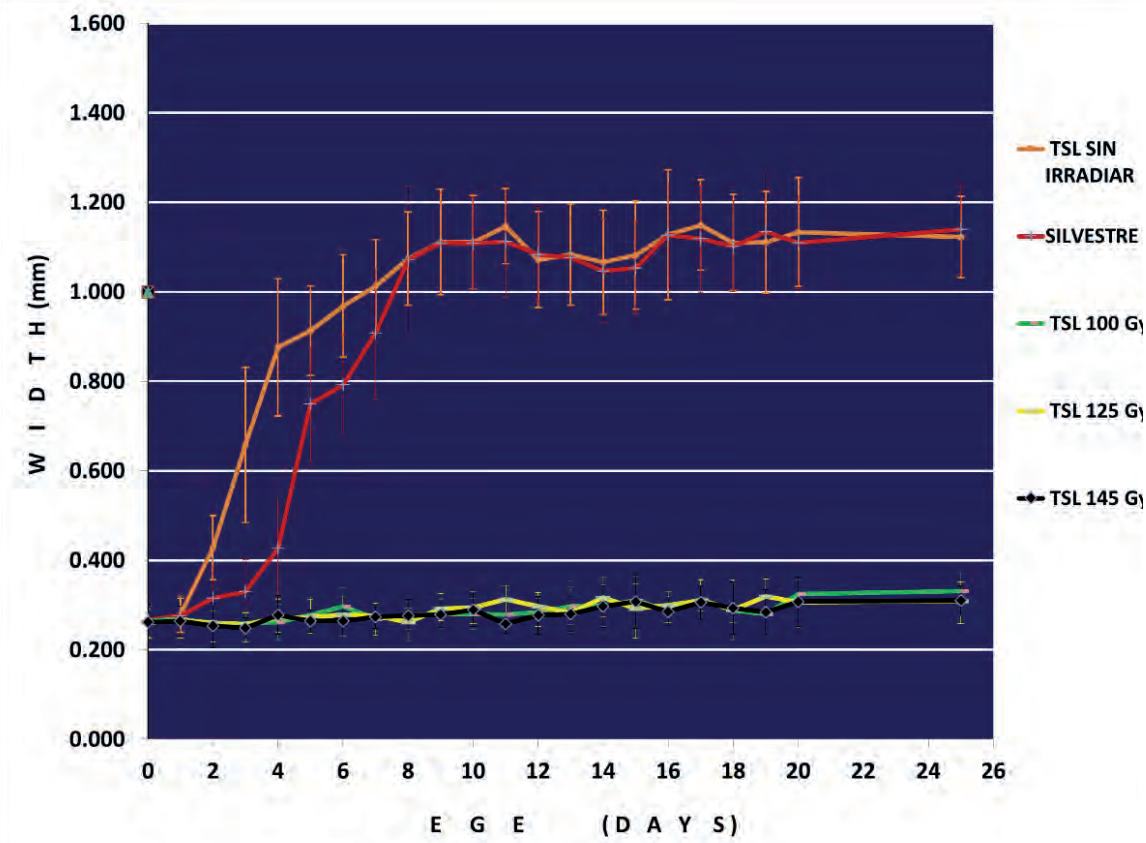

Figure 10. Ovary width of the following *C. capitata* flies: non-irradiated TSL flies and wild and TSL flies irradiated at 100, 125 or 145 Gy from emergence (day 0) to 25 days old. Vertical bars represent the standard error. For each age n = 15.

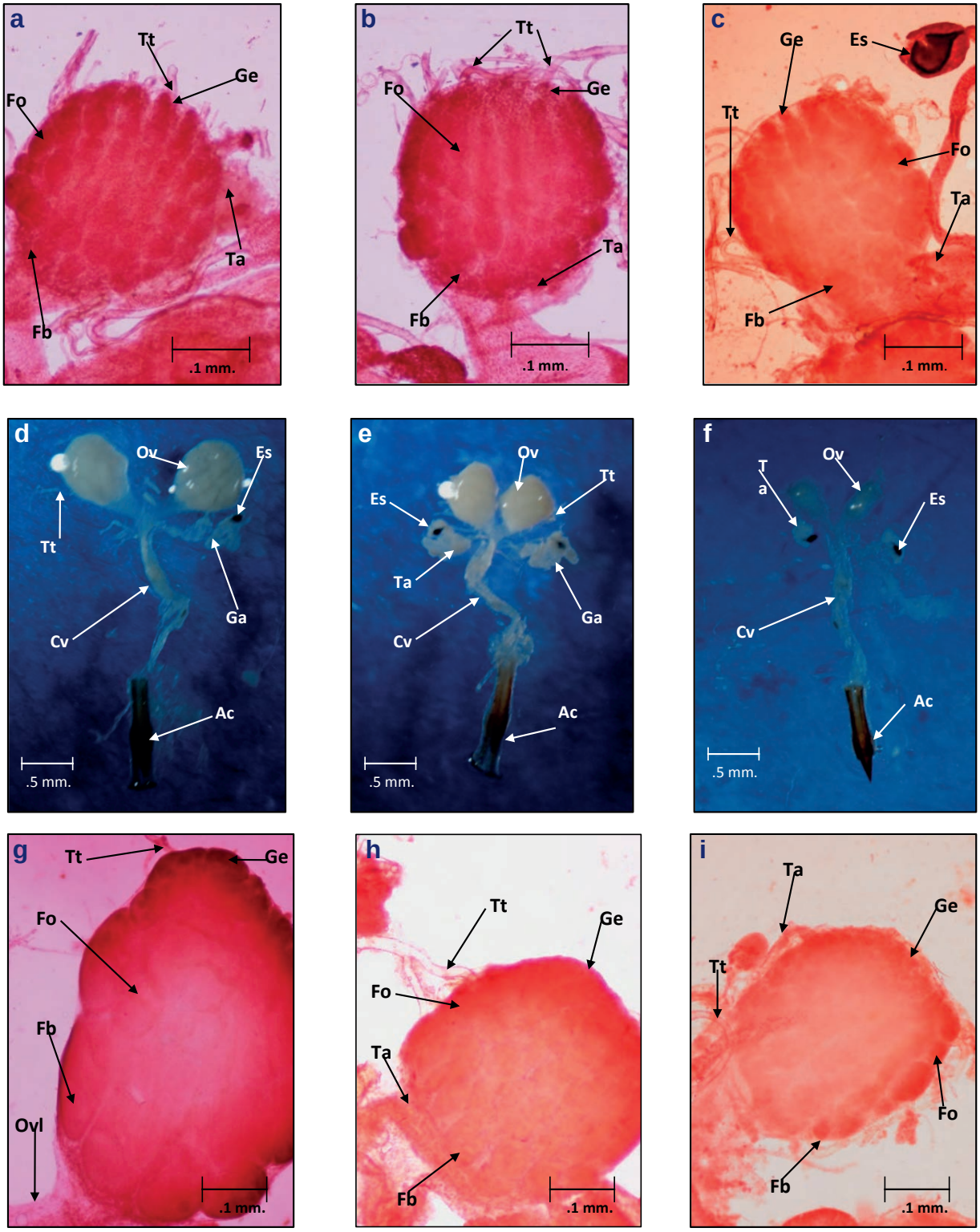

Figure 11. a) Non-irradiated stained TSL flies at day 1; b) Wild stained flies at day 1; c) Stained TSL flies irradiated with 100 Gy at day 1; d) Non-irradiated TSL flies at day 2; e) Wild flies at day 2; f) Stained TSL flies irradiated with 125 Gy at day 2; g) Non-irradiated, stained TSL flies at day 2; h) Wild stained flies at day 2; and i) Stained TSL flies irradiated with 145 Gy at day 2: Ac= Aculeo, Cv= Vaginal duct, Es= Spermathecae, Fb= Basal follicle, Fo= Ovarian follicle, Ga= Accessory gland, Ge= Germarium, Gr= Rectal gland, Ov= Ovary, Ovl= Lateral oviduct.

Table 3. Number of mature oocytes per ovary from non-irradiated TSL flies and wild and TSL flies irradiated with 100, 125 or 145 Gy (mean ± SD). For each treatment, n = 15 females per day and both ovaries

AGE (DAYS)	Mature oocytes NON-IRRADIATED TSL		WILD		TSL 100 Gy	TSL 125 Gy	TSL 145 Gy
0	0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1	0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2	0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3	1.2 ± 2.7	a	0.1 ± 0.7	b	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
4	10.1 ± 7.5	a	0.8 ± 2.9	b	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
5	14.4 ± 4.9	a	8.0 ± 5.7	b	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
6	11.3 ± 5.1	a	9.1 ± 6.0	a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
7	12.0 ± 4.7	a	9.2 ± 8.6	a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
8	14.2 ± 5.9	b	19.8 ± 9.9	a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
9	16.5 ± 5.0	a	18.0 ± 6.8	a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
10	15.9 ± 5.9	a	18.2 ± 10.8	a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
11	16.6 ± 6.0	a	20.3 ± 10.5	a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
12	17.3 ± 6.3	b	26.3 ± 8.9	a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
13	20.2 ± 8.1	a	18.0 ± 5.5	a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
14	18.5 ± 5.0	a	20.8 ± 8.3	a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
15	21.9 ± 10.9	a	18.7 ± 5.4	a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
16	28.3 ± 10.7	a	23.0 ± 6.9	b	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
17	21.2 ± 12.8	a	20.9 ± 11.1	a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
18	23.9 ± 8.6	a	16.6 ± 6.3	b	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
19	26.8 ± 8.2	a	16.4 ± 5.8	b	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20	21.5 ± 10.8	a	12.0 ± 3.6	b	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
25	20.7 ± 9.7	a	12.3 ± 2.7	b	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

For each value, followed by the same letter in the same row means they are not statistically different (Fisher's exact test, P <0.05).

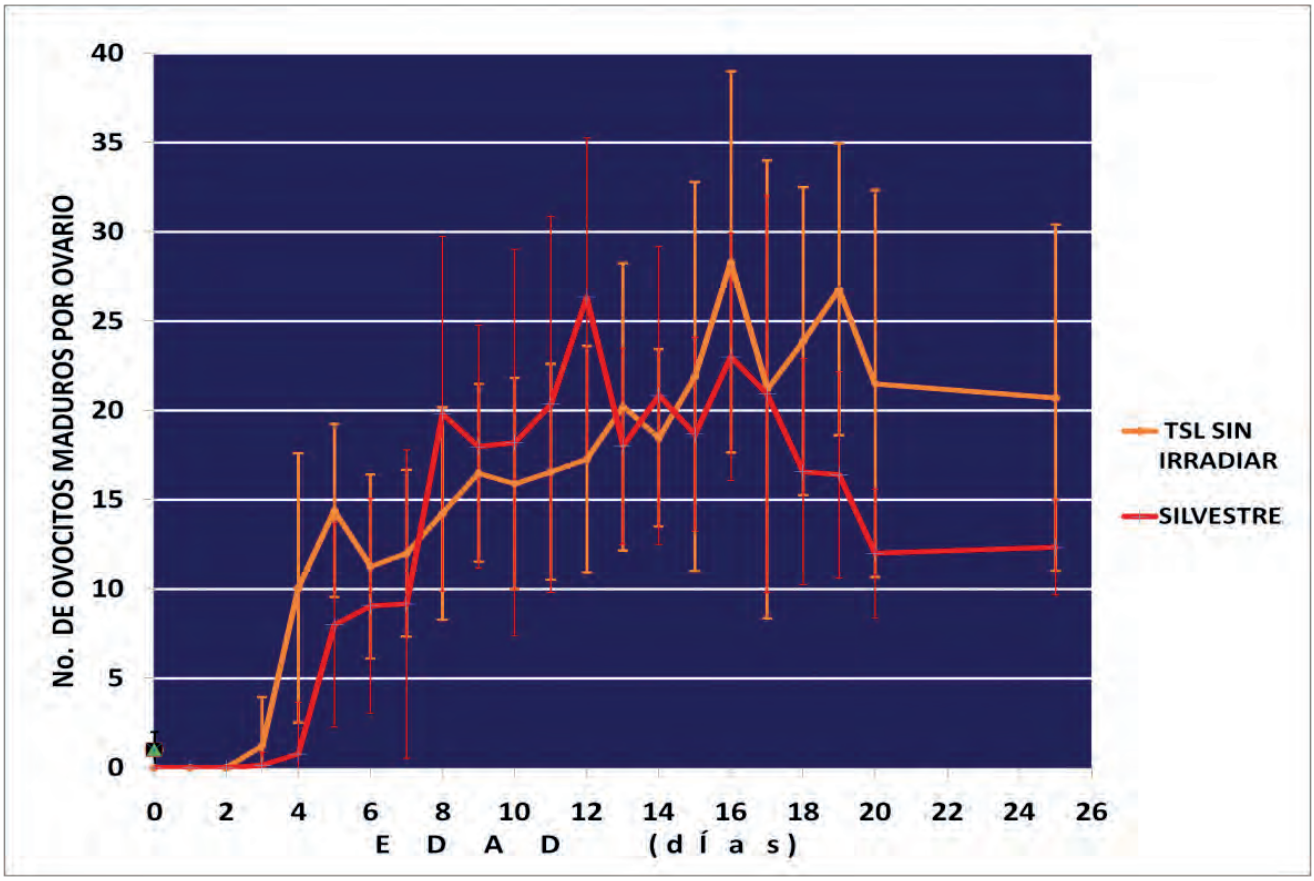

Figure 12. Number of mature oocytes per ovary of C. capitata of irradiated TSL flies and wild and TSL flies irradiated with 100, 125 or 145 Gy from emergence (day 0) to 25 days old. Vertical bars represent the standard error. For each age, n = 30 ovaries.

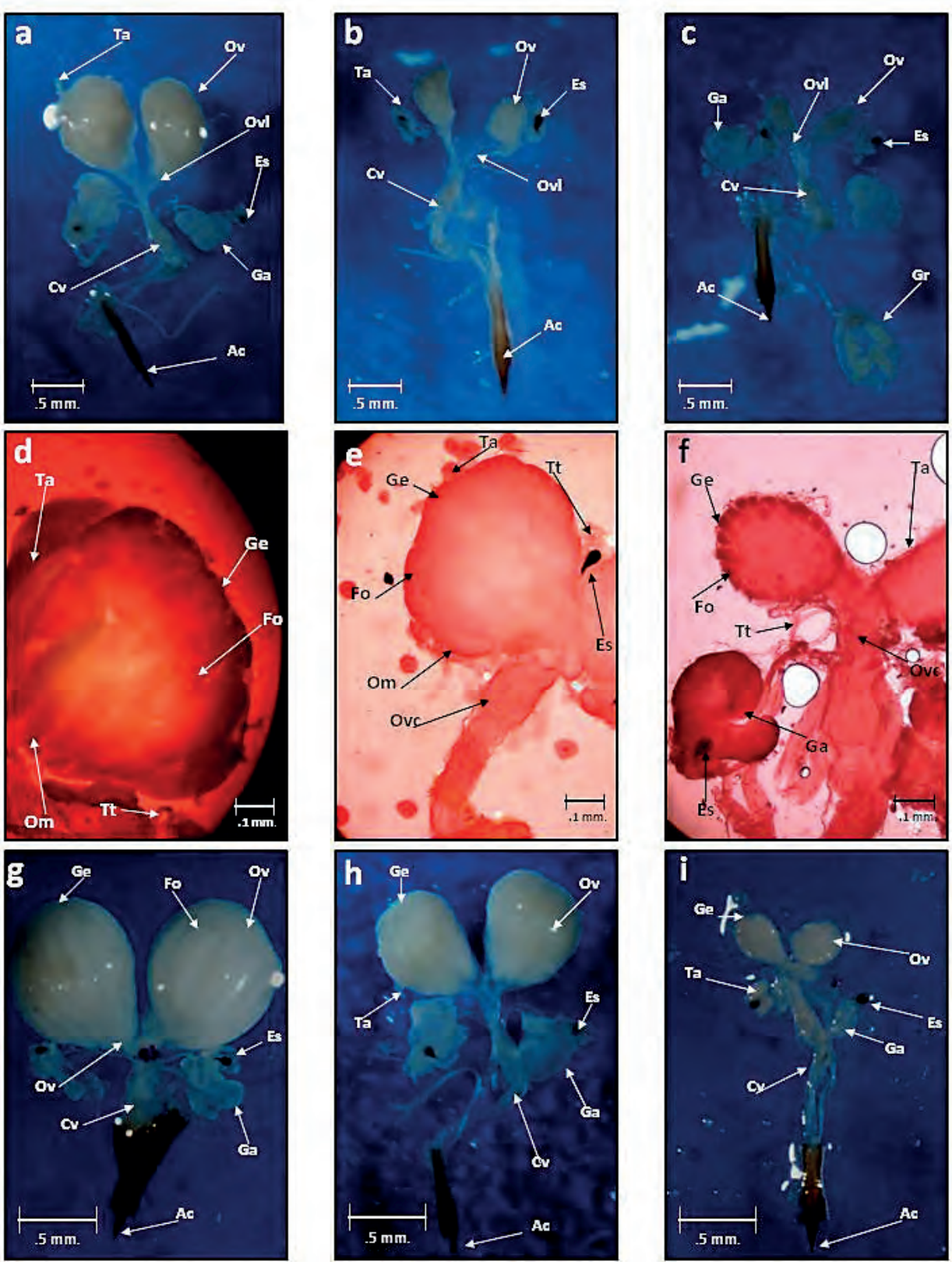

Figure 13. a) Non-irradiated TSL flies at day 3; b) Wild flies at day 3; c) TSL flies irradiated with 100 Gy at day 3; d) Non-irradiated stained TSL flies at day 3; e) Wild flies stained at day 3; f) Stained TSL flies irradiated with 125 Gy at day 3; g) Non-irradiated TS flies at day 4; and h) Wild flies at day 4; i) TSL 145 Gy at day 4: Ac= Aculeo, Cv = Vaginal duct, Es= Spermathecae, Fo= Ovarian follicle, Ga= Accessory gland, Ge= Germarium, Gr= Rectal gland, Om= Mature oocyte, Ov= Ovary, Ovl= Lateral oviduct, Ta = Adipose tissue, Tt= Tracheal tube.

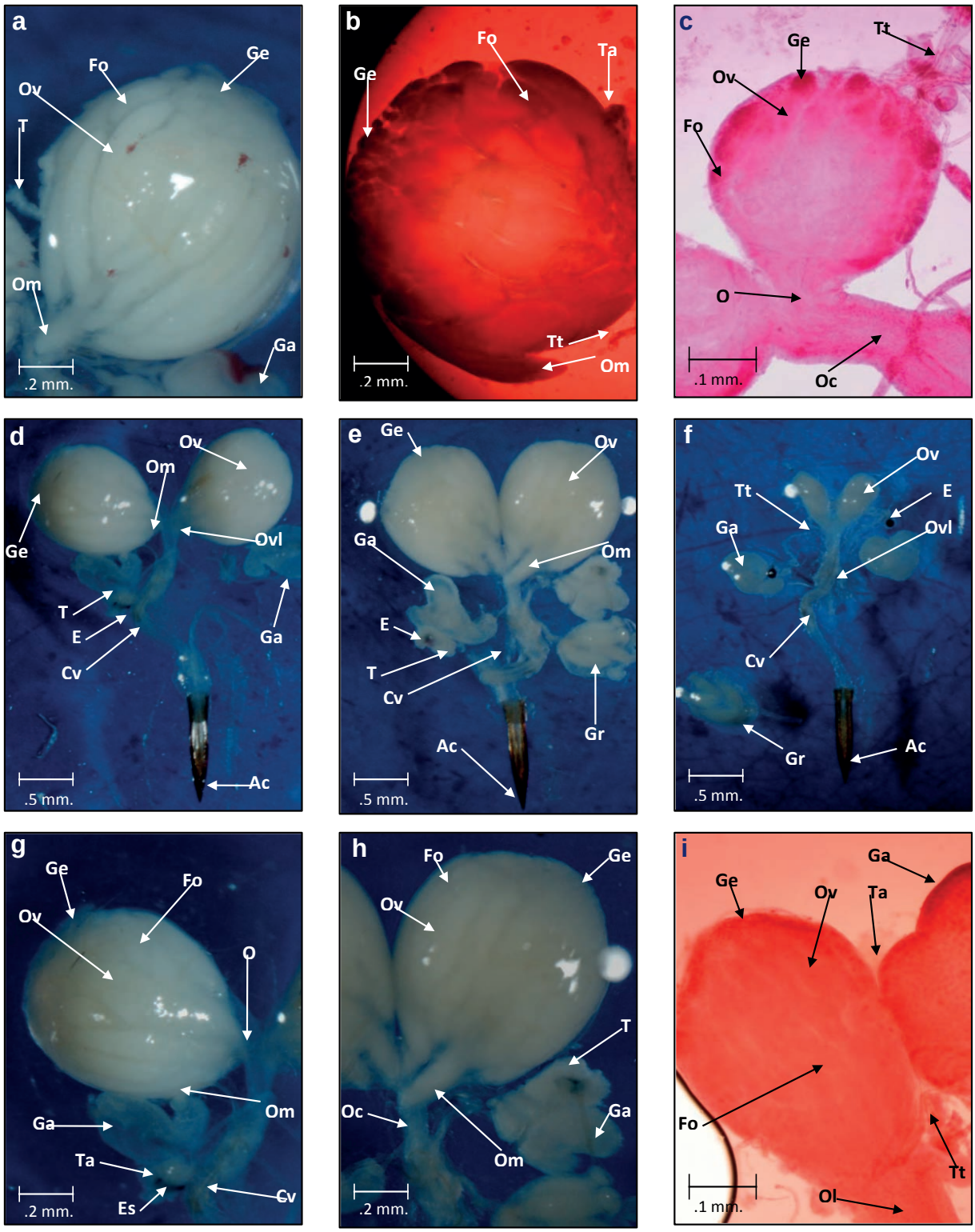

Figure 14. a) Non-irradiated TSL flies at day 4; b) Wild stained flies at day 4; c) Stained TSL flies irradiated with 100 Gy at day 4; d) Non-irradiated TSL flies at days 5-8; e) Wild flies at days 5-8; f) TSL flies irradiated with 125 Gy at days 5-8; g) Non-irradiated TSL flies at days 5-8; magnified; h) Wild flies at days 5-8, magnified; and i) Stained TSL flies irradiated with 145 Gy at days 5-8: Ac= Aculeus, Cv = Vaginal duct, Es= Spermathecae, Fo= Ovarian follicle, Ga= Accessory gland, Ge= Germarium, Gr= Rectal gland, Om= Mature oocyte, Ov= Ovary, Ovl= Lateral oviduct, Ta = Adipose tissue, Tt= Tracheal tube.

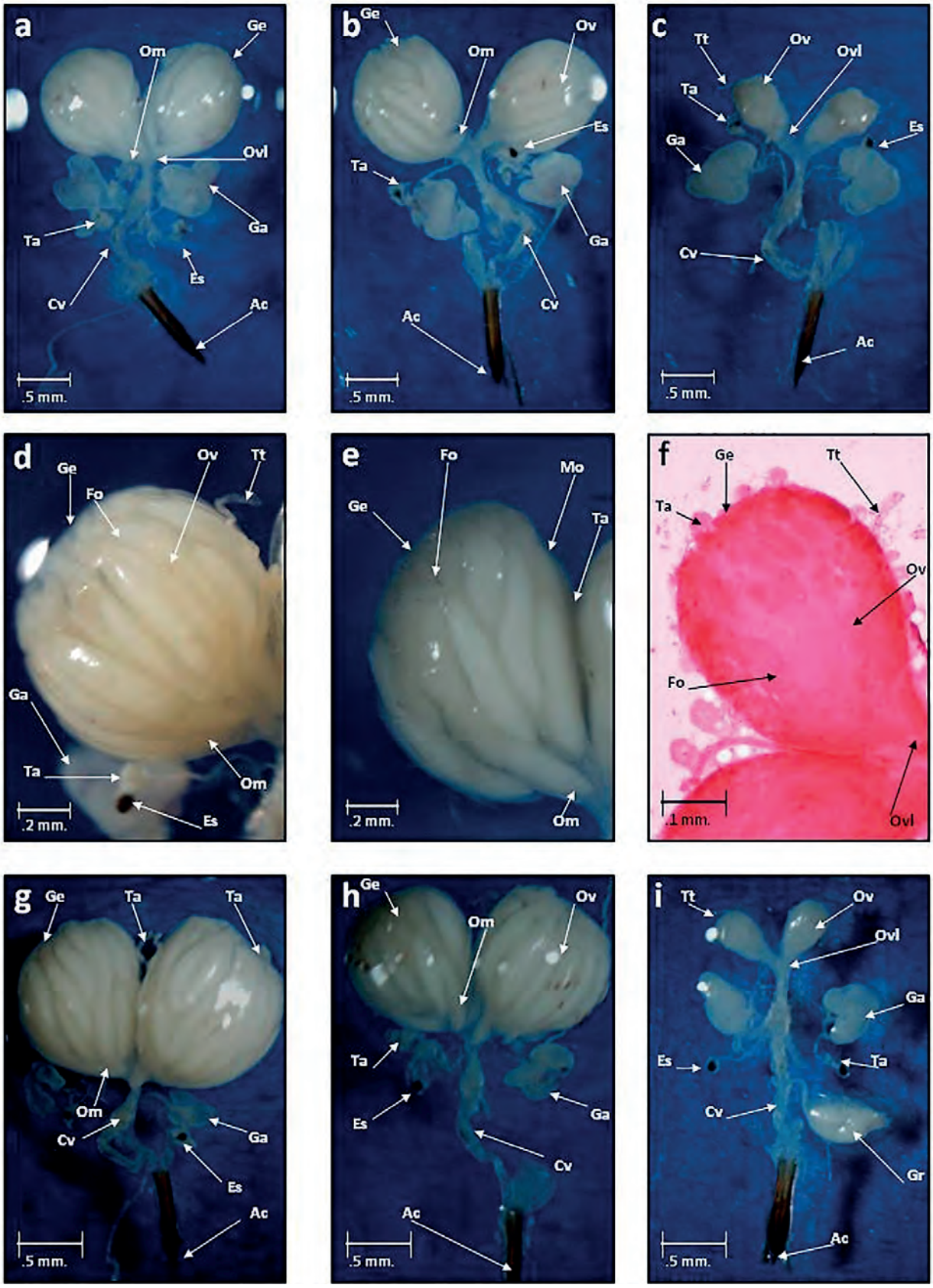

Figure 15. a) Non-irradiated TSL flies at days 8-20; b) Wild flies stained at days 8-20; c) TSL flies irradiated with 100 Gy at days 8-20; d) Non-irradiated TSL flies at days 8-20, magnified e) Wild flies at days 8-20, magnified; f) Stained TSL flies irradiated with 125 Gy at days 5-8; g) Non-irradiated TSL flies at day 25; h) Wild flies at day 25; and i) TSL flies irradiated with 145 Gy at day 25: Ac= Aculeo, Cv = Vaginal duct, Es= Spermathecae, Fo= Ovarian follicle, Ga= Accessory gland, Ge= Germarium, Gr= Rectal gland, Mo= Ovarian membrane, Om= Mature oocyte, Ov= Ovary, Ovl= Lateral oviduct, Ta = Adipose tissue, Tt = Tracheal tube.

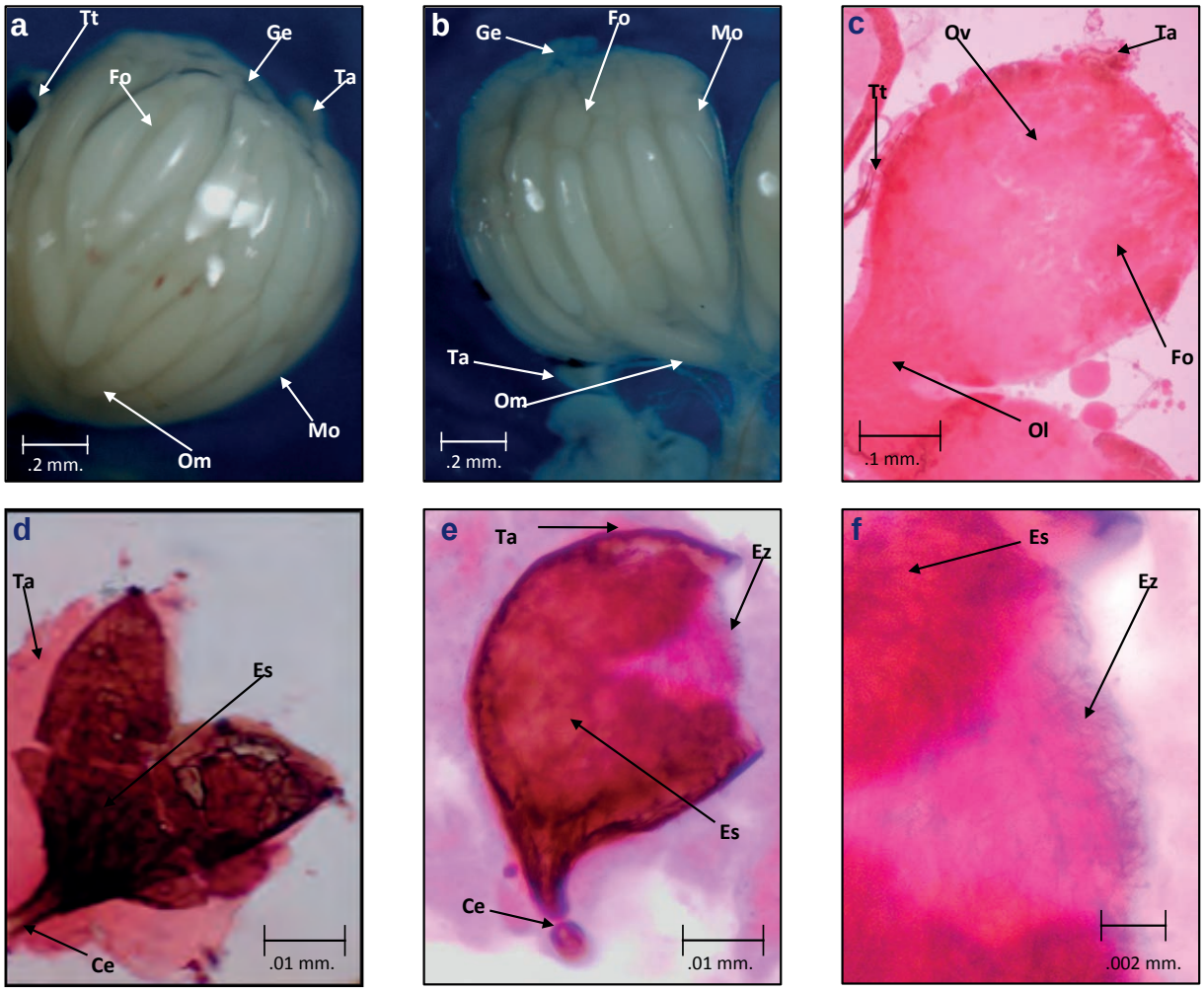

Figure 16. a) Non-irradiated TSL fly ovary at day 25; b) Wild fly ovary at day 25; c) Stained TSL flies ovary irradiated with 100 Gy at day 25; d) Wild female spermathecae at day 4; e) Wild female spermathecae at day 10, with sperm; and f) Wild female spermathecae at day 10. Ce= Spermathecal duct, Es= Spermatheca, Ez= Sperm, Fo= ovarian follicle, Ge= Germario, Mo= Ovarian membrane, Om= Mature oocyte, Ov= Ovary, Ol= Lateral oviduct, Ta= Adipose tissue, Tt= Tracheal tubes.

4. Determination of fertility or sterility **of males through cytohistological** analysis of the testes

If unmarked males are captured in the area where sterile TSL flies are released, these might be either wild or sterile males.

4. 1 Reproductive system of males

The reproductive system of males produces sperm for egg fertilization. The reproductive system of Medfly males is similar to other tephritidae. The terminology used here is based on the work of Matzuda (1976) and McAlpine (1981), and for internal structures particularly the work of Hanna (1938) and Guillen (1983). The male reproductive system consists of the following structures (Figures 17 and 18).

Testicles. These are of mesodermal origin, ovoid in form at full sexual maturity. The membrane that protects them, a simple epithelial layer, is covered with an inter-connective tissue layer forming a capsule in which all of the cells involved in spermatogenesis are enclosed. In young flies (0 – 4 days old), this tissue is pale yellow becoming more intense and brighter yellow with age. In sexually mature males, the testis can be divided into four zones. The germarium or growth zone, where spermatogonial cells and primary and secondary spermatocytes are found, is located in the apical regional of the testis. The next is the zone of spermatids; these are located in membranous sacs or bladders at different developmental stages in a synchronized process to become sperm. In the intermediate region is the sperm packs zone (or sperm bundles zone), formed by groups of filamentous-shaped sperm. The last is the basal zone of the testis, where the seminal vesicle is found. In mature males, this last zone has large quantities of accumulated free sperm that have matured and dropped from sperm packs (bundles) and, as in the sperm bundles zone, they are entangled and filamentous; distinguishing the head from the tail is difficult.

Vas deferens. These are tubes connected to the basal extreme of the basal zone of each testicle and extend into the ejaculatory duct in its first section, which in turn communicates with the ejaculatory apodeme.

Ejaculatory apodeme. Also called the sperm pump, it has a rounded pear-shaped form with a narrow base, consisting of two highly sclerotic structures of cuticular nature. This organ is believed to cause pressure from muscle contractions by pushing the seminal fluid containing mature sperm to the terminal end of the ejaculatory duct.

Ejaculatory duct. This long duct consists of three sections. The first, the most anterior and widest, is where the vas deferens of each testis and the ducts of the accessory glands merge. The second, is the middle section, is shorter than the first and continues through the base of the ejaculatory apodeme to reach the third section. This distal end is slightly thinner than the previous sections and connects to the aedeagus.

Accessory glands. These are present in pairs and are small structures in the form of elongated tubes with translucent appearance. The glands are connected to the first section of the ejaculatory duct to help with their secretions in the development of seminal fluid and maintenance of the sperm.

Aedeagus. The final structure of the male reproductive system is formed by the aedeagus. This organ is attached at its distal end to the last section of the ejaculatory duct and consists of a thin sclerotized tube. Along with other structures in the terminalia of the male, it forms most of the intromittent organ, which is anatomically and physiologically adapted for copulation and sperm transfer to females.

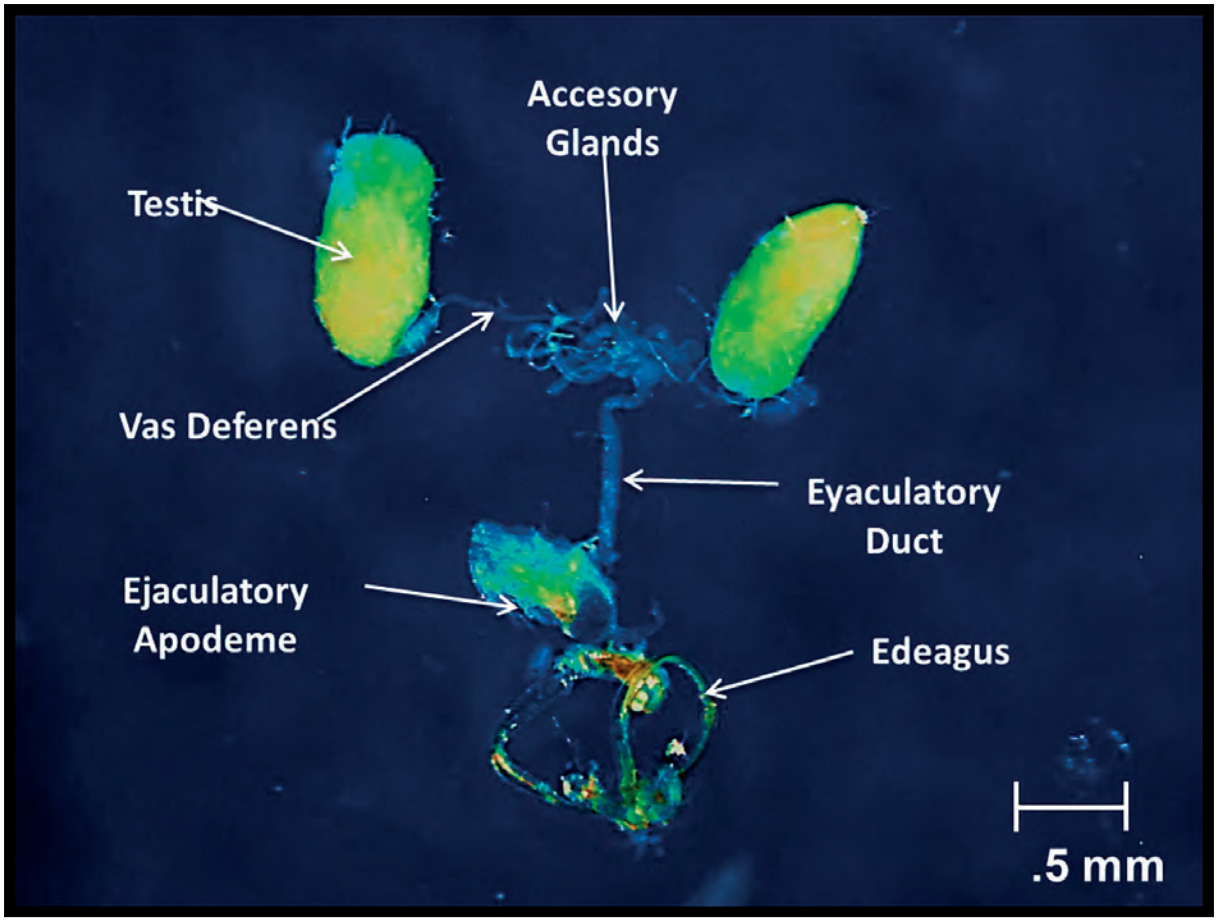

Figure 17. Male reproductive system of a wild Medfly, 9 days old.

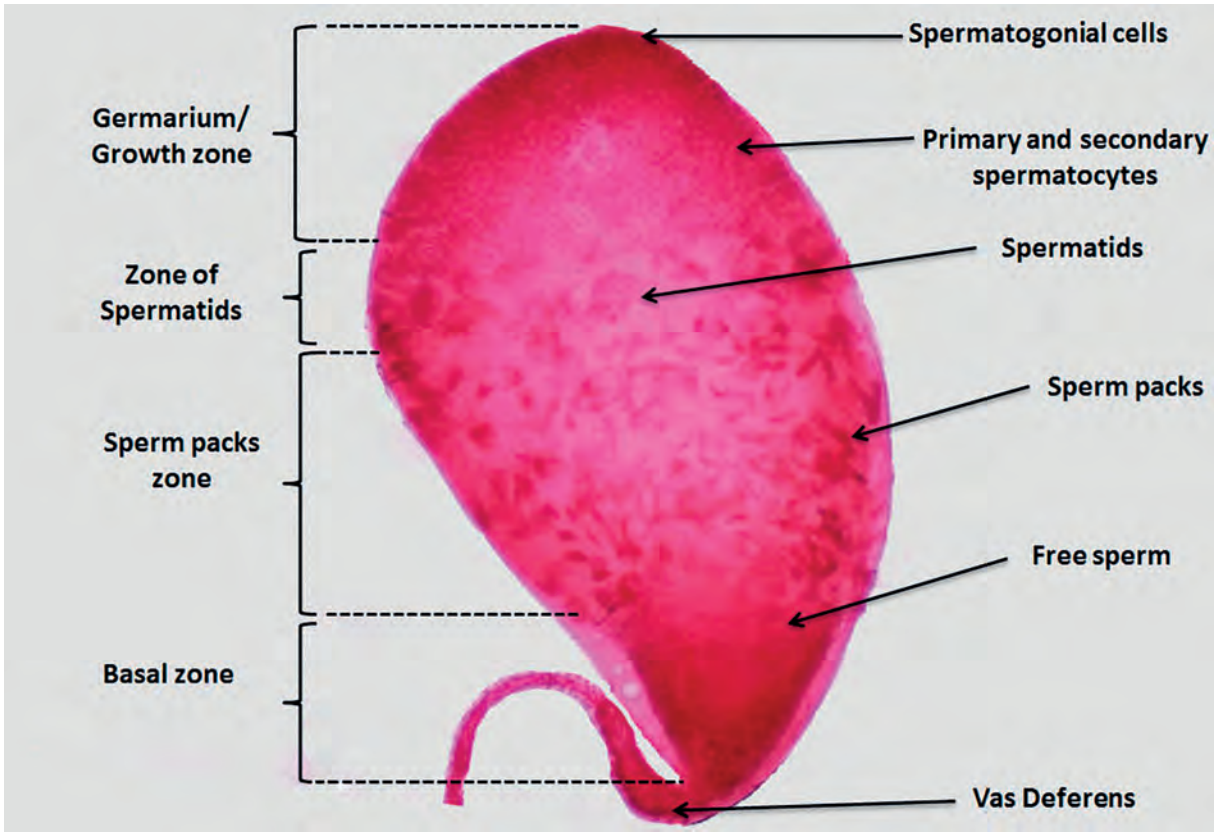

Figure 18. Testis of a wild male Medfly 10 days old, indicating the cells of the normal developmental stages of spermatogenesis.

4.2 Sexual maturation of males and effect of irradiation on the testes

Unlike females, sexual maturation in males has already begun at the time of fly emergence. In males, since the very first day, small amounts of free sperm in the testes base can be detected in quantities that increase with age.

Spermatogenesis of Medfly is similar to other diptera. Normally, the primary spermatogonial cells in the germarium or growth zone, located at the apical region of the testes, become secondary and produce primary spermatocytes after successive mitotic divisions, which divide meiotically to produce secondary spermatocytes that later will be transformed into spermatids. By continuing spermatogenesis, spermatids give rise to packs (bundles) of tightly bound sperm; as they mature, they break the wall of the sac containing them and once free they remain at the base of the testis or, in some cases, in the vas deferens ready to be transferred to the females during mating.

Between the fourth and fifth days after emergence, the histological differences between testes of wild and mass-reared non-irradiated TSL, and mass-reared irradiated males are beyond question; therefore, differentiation between them is highly reliable. As the wild and non-irradiated male flies continue with the normal process of spermatogenesis, the irradiated males show clear mitotic inhibition, cell death and disintegration, which gradually produce a complete disruption of the cells involved in spermatogenesis. Therefore, the possibility of generating new spermatogonia cells is eliminated, and only spermatids cells that were under development when irradiation was carried out become dense abnormal sperm. These abnormal sperm gradually accumulates in the base of the testes until nearly completely filling the testicular cavity in the last days of the life of males (Figure 23 c, f and i).

Based on the characteristics of spermatogenesis, a practical guideline for rapid differentiation of non-irradiated and wild male flies can be:

1. Non-irradiated TSL males from emergence to 3 or 4 days old show signs of being at early sexual maturity. After 4 days old, they can be considered of being at advanced sexual maturity.
2. Wild males from emergence to 5 or 6 days old show features of being at early sexual maturity. After 6 days old, they can be considered of being at advanced sexual maturity.
3. However, for a more complete description of testicle features and irradiation damage to diagnose the sterility or fertility condition of males it is recommended to go to **Section 4.4**.

4.3 Determination of the sterile or fertile condition of males

Dissection of testes

The male abdomen is placed in a vial with saline solution (**Section 2.6** and **Appendix 2**). The abdomen is then placed on a microscope slide in a dorsal position so that the last abdominal segments point towards the observer. Using magnification lenses (10x to 30x) of a stereomicroscope and extra fine-tipped entomological tweezers No. 5, dorsal and ventral abdominal segments are carefully separated. Once the interior of the abdomen is exposed, the males' reproductive system is dissected very delicately to avoid rupturing its different structures (testes, accessory glands, ejaculatory apodeme, etc.). The dissection should be performed with the abdomen immersed in distilled water to avoid the dehydration of the structures. In order to focus on the testes, any remaining tissues that are not of interest to the study should be removed.

The testes are located at the lower lateral ends of the abdomen's last segments; these are ovoid with shades ranging from pale yellow to deep yellow. Once located and isolated from other tissues the testes are transferred to another microscope slide; one or two drops of aceto-orcein are added (**Appendix 4**). 3 – 4 minutes are required to get the cells involved in spermatogenesis stained. In the germarium or growth zone, aceto-orcein stains the cells of non-irradiated and sexually mature males with a more intense

crimson red than the other cells due to the positive reaction of the dye with the chromatic material of the nucleus. The clusters of spermatids in the spermatids zone usually have a triangular or ovoid-shaped pattern. Once the cells have been stained, the testes are covered with a coverslip for observation, using the 10x objective lens to locate the testicles and the 40x and 100x lenses for cytohistological analysis.

If a permanent mounting is desired, dissected testes and adjacent structures should be transferred to a plain slide and covered with mounting medium (Canadian balsam, Hoyer liquid, or Entellan). If the microscopy equipment has a digital camera installed on it, it is worthwhile to take microphotographs at different magnifications to have a long-lasting record of the mounting.

Sterility or fertility of males

The following criteria should be considered:

1. **If the testes show defined spermatogenesis, the fly should be determined as fertile**. The different zones become defined and can be identified easily. The different cells in the germarium or growth zone are intensely stained and maintain a uniform distribution without large intercellular spaces. Spermatids with well-defined cysts are observed between the growth zone and the sperm packs zone (Sperm bundles zone). Sperm bundles are now abundant, although many are loose, as they are releasing free sperm that accumulate at the base of the testes.

2. **If the testes show evident histological damage, the fly should be determined as sterile**. This damage is observed as a clear reduction in the quantity of spermatogonial cells, and those that remain are very weakly stained, with large intercellular spaces observed. Spermatids are scarce and located in the growth zone, and sperm bundles and some free sperm can be found. In the sperm pack zone, spermatid size is also reduced by the increased release of free sperm into the seminal vesicle.

If sterile flies are released at uniform ages, once they are under advanced sexual maturity (at least 4 days after emergence), the expected error for sterility determination is zero, provided that the captured specimens are in good condition. However, if the testis shows undefined spermatogenesis, then the final determination can be made based on the technical criteria presented in **Section 4.4**.

4.4 Testes maturation in wild and non-irradiated VIENNA Medfly TSL males compared with VIENNA Medfly TSL males irradiated one day before emergence with 100, 125 and 145 Gy

Day 0 (Emergence). (Fig. 19a-c, 20, 21 and Tables 4, 5).

In newly emerged males of all groups, spermatogenesis has already begun. The incipient formation of the germinal zone can be observed in the apical region, with developing and well-defined spermatogonial cells and primary and secondary spermatocytes occupying approximately 10 to 15% of the length of the testis. Immediately after the growth zone, although not abundant, spermatid groups can be observed arranged in both the central and side walls. In the sperm pack area (sperm bundle area), the groups can be observed tightly packed in small quantities. A few can be found to be weaker than the rest, and these are releasing small amounts of free sperm into the seminal vesicle or base of the testis that is located close to the connection with the vas deferens. These features are similar in all groups, although a slightly larger amount of free sperm can be found in the testes of some irradiated males, while it is not possible to establish clear differences in the characteristics of the testes of non-irradiated TSL and wild males.

The appearance of the testes of all groups at this age is mostly rounded or globe-like with a pale yellow colour, and the cover or protective case is very thin and fragile upon contact with the fine-tipped dissecting tweezers. Although smaller testes can be found at this age, generally the morphometric data do not indicate differences between the non-irradiated and irradiated flies. However, differences can be observed between the testes size of non-irradiated TSL flies and wild flies. The non-irradiated have an

average length of 0.574 ± 0.058 mm and width of 0.351 ± 0.042 mm, which is slightly greater than the average size of the testes of wild flies, which measure 0.466 ± 0.042 x 0.313 ± 0.038 mm.

Days 1-2. (Fig. 19d-i, 20, 21 and Tables 4, 5).

During days 1-2, all of the testes of the different flies increase in size; however, larger dimensions can be observed on day 2 in the testes of non-irradiated TSL flies, which are 0.635 ± 0.049 mm long and 0.326 ± 0.036 mm wide, compared to smaller testes of wild flies at 0.466 ± 0.042 x 0.313 ± 0.038 mm. The testes of the three groups of irradiated flies were not statistically different in size, which ranged from 0.582 to 0.563 long and from 0.276 mm to 0.305 mm wide.

Although there are morphometric differences between the testes of non-irradiated flies and those of wild males, the histological features generally observed on days 1-2 do not allow for the clear differentiation among the different flies. Minor differences are observed compared to earlier ages, with only a slight increase in the growth zone of up to 16-20% of the total length of the testes in non-irradiated TSL flies and 15-18% in the other groups.

The size of the basal part of the testis, or seminal vesicle, also increases slightly by approximately 5-10% in non-irradiated TSL flies due to the formation of more sperm bundles. This trend indicates an advance in sexual maturity compared to other treatments in which a maximum of up to 5% free sperm pooling and fewer sperm bundles are observed.

Days 3-4. (Fig. 20, 21, 22a-f and Tables 4, 5).

At this age, the testes of all males continue to increase in size and become more elongated with no important differences. Only the wild flies have a slightly smaller size, reaching an average on day 4 of 0.570 ± 0.037 x 0.296 ± 0.032 mm; the other flies range between 0.667 ± 0.029 and 0.683 ± 0.041 mm long and between 0.277 ± 0.035 and 0.323 ± 0.023 mm wide. Although the continuity of spermatogenesis in the testes of non-irradiated wild and TSL males and the start of irradiation damage in the irradiated groups can be observed on days 3-4, it is not possible to clearly and definitively determine important differences that allow for the differentiation of both groups of flies.

On day 4, the testes of at least 40% of the specimens of the non-irradiated TSL strain may have more advanced spermatogenesis features, as the germarium or growth area reaches up to 25% of the total length of the testes. An increase in sperm bundles can be detected, which release greater amounts of free sperm at the base of the testes and occupy up to 20% of the length of the testis; in the other flies, characteristics similar to those observed on day 2 are presented. On day 4, the testes of 25% of males have characteristics similar to the younger flies.

In the irradiated flies, only 20% of all specimens show early signs of radiation damage; however, this damage is hardly distinguishable from non-irradiated specimens. Damage is characterized by an increase in the intercellular space of the growth zone, an absence or decrease in the number of spermatids and a fair number of sperm bundles releasing free sperm at the base of the testes and reaching up to 20% of their length. The remaining specimens have characteristics similar to those observed on day 2.

Day 5. (Fig. 20, 21, 22g-i, 23a-c and Tables 4, 5).

From this day, significant changes occur in both the group of non-irradiated males (wild and TSL males) and in the group exposed to the three irradiation doses. The testes of non-irradiated males have mostly ovoid forms. In non-irradiated TSL males, the testes are significantly larger than in the wild males (Tables 4 and 5); however, the non-irradiated flies are not different from the irradiated group, and both have larger sizes with an average for all groups of 0.688 ± 0.031 mm long and 0.287 ± 0.033 mm wide. From day 5 onward, the irradiated flies show a dramatic decrease in size.

During spermatogenesis in the non-irradiated flies, the different zones become defined and can be identified easily in the wild and in non-irradiated TSL males. In both groups, the growth zone reaches

25 to 30% of the total length of the testes, and the different cells in this area are intensely stained and maintain a uniform distribution without large intercellular spaces. A slightly more pronounced homogeneous growth in non-irradiated TSL flies maintains a developmental advantage as compared to the wild flies. Spermatids with well-defined cysts are observed between the germination area and sperm bundles. Sperm bundles are now abundant, although many are weak, as they are releasing free sperm that accumulate at the base of the testes. This free filiform and entangled sperm reach up to one quarter of the length of the testes.

In the irradiated flies, histological damage is evident, and a clear distinction can be observed as compared with the non-irradiated flies. This damage is observed as a clear reduction in the quantity of spermatogonial cells with those cells that remain are very weakly stained. Irradiation causes nuclear pyknosis in spermatocytes and spermatids, which in turn causes cell necrosis, and it is possible to observe large intercellular spaces. Spermatids are scarce and located in the germination area and sperm bundles, and some free sperm can be found. In the sperm pack zone, spermatid size is also reduced by the increased release of free sperm into the seminal vesicle, which increases considerably in size to between 35 and 40% of the total size of the testes in the different groups, which is almost double the size at 3-4 days.

Days 6-10. (Fig. 20, 21, 23d-i, 24c and Tables 4, 5).

At this stage of development, the size of the testes of non-irradiated TSL flies ranges from 0.665 ± 0.036 to 0.695 ± 0.029 mm long and 0.295 ± 0.030 to 0.315 ± 0.033 mm wide, which is larger than the size of the testes of the wild flies, which reach a maximum on day 7 of 0.655 ± 0.061 x 0.313 ± 0.038 mm. In addition to the difference in size, the testes of non-irradiated TSL flies may exhibit, at least until day 8, slight but noticeable progress in spermatogenesis as compared to the wild flies, as characterized by an increase in the germination zone, which occupies slightly more than 30% of the length of the testicles with a similar proportion permanently occupied by free sperm in the basal region. These parameters are slightly lower in the testes of wild males until day 8, but afterwards, the differences become smaller between the two groups up to day 10, exhibiting minimal histological differences and maintaining morphologically ovoid forms. Another feature at days 6-10 in both non-irradiated types of male is the beginning of a slight separation of the cover of the testicles at the seminal vesicle level, which remains approximately between 25 and 30%.

There is a clear decrease in testes size in all three groups of irradiated flies, with few significant differences among them but with a clear difference from the non-irradiated flies. The testes irradiated at different doses begin to acquire elongated shapes mainly due to necrotic cell death caused by irradiation and subsequent absorption, thereby causing atrophy of the testes, which on day 10 can reach an average size of 0.539 ± 0.041 long and 0.202 ± 0.034 mm wide.

In the apical region, irradiation damage is already very clear, and spermatogonial cells disappear almost completely; if some are found, they are very poorly stained. Irradiation causes intense pyknosis and cell destruction in spermatocytes, and spermatids have also disappeared or only a few weak cysts remain. The result is large intercellular spaces that are occasionally observed to be occupied by remnants of sperm bundles and, in some cases, by free sperm that migrate to the apical region of these testes. In the sperm packed zone, the bundles are in poor shape until day 8; on subsequent days, they disappear because they have weakened and released free sperm, which are deposited at the base of the testis. The seminal vesicle is filled as the male's age, reaching up to 45 to 50% of the length of the testes on day 6, 50 to 55% on day 7, 55 to 65% on day 8, 65 to 75% on day 9 and up to 80% on day 10. In the TSL males irradiated with 145 Gy, it is possible to observe a slightly later release of sperm.

In the irradiated flies, there is a separation of the testicular sheath, which is directly related to the amount of sperm released; therefore, this separation is greater in the irradiated flies than in the non-irradiated groups of the same age.

Days 11-15. (Fig. 20, 21, 24d-i and Tables 4, 5).

In this period, the non-irradiated flies are stable in size and in the development of spermatogenesis, and a constant production of sperm is observed, although as in previous days, the average size of the testes of non-irradiated TSL flies is larger than those of the wild flies, ranging between 0.671 ± 0.047 and 0.689 ± 0.072 mm long by 0.279 ± 0.036 and 0.295 ± 0.031 mm wide. The wild fly testes remain between 0.621 ±0.059 and 0.643 ±0.031 mm long by 0.275 ± 0.028 and 0.291 ± 0.031 mm wide. Histologically, it is difficult to observe differences between the TSL and the wild males. Three well-defined regions along the testicles are easily distinguishable, each occupying approximately one third the length of the testes. The first is the apical region or growth area, in which new spermatogonial cells are produced to generate new primary and secondary spermatocytes with homogeneous development and are intensely and uniformly stained. In the bottom of this area are located spermatids with synchronous growth that are becoming sperm and look similar to fingerprints. The second region in the middle is the sperm packed zone that continues to differentiate and release free sperm to the seminal vesicle, which occupies the final third of the testicle. In some testicles, it is possible for the latter area to occupy up to 40 to 45% of the length, which is most likely related to poor or a lack of sperm transfer to females through the absence of mating.

In the irradiated flies, it is possible to observe a clear and rapid decrease in testicle size caused by the continued death and absorption of cells produced by irradiation. On days 11-15, the testes have an elongated, irregular kidney shape. The morphometric data of all fly groups range between 0.551 ± 0.033 and 0.465 ± 0.029 mm long and 0.291 ± 0.031 and 0.295 ± 0.031 mm wide, with little or no significant differences among them, but with differences that are significant compared with the non-irradiated flies (Tables 4 and 5). Two well-defined and delimited regions along the testicles can be easily distinguished at least until days 11 and 12. The first is in the apical region, where severe histological damage appears with a clear destruction of the growth zone. It is not possible to detect spermatogonial cells, spermatocytes or spermatids; the cellular spaces left by the disintegration of cells are very large; necrotic areas can be distinguished from the effect of pyknosis; and occasionally, it is possible to find remnants of bundles and free sperm in the most apical zone in this area. On days 11 and 12 the rest of the testicular cavity is occupied by up to 90 to 95% by dense abnormal free sperm, however, from this day free sperm may occupy the entire testicular cavity.

Days 16-20. (Fig. 20, 21, 25a-f and Tables 4, 5).

In the non-irradiated males, sperm production continues steadily, a clear sign of full sexual maturity. If these males copulate, it may affect their testis sizes because they alternate periods of sperm transfer and accumulation in the seminal vesicle. The testes of non-irradiated TSL flies may be larger compared to those of the wild flies until day 19, ranging between 0.690 ± 0.060 and 0.702 ± 0.054 mm long and 0.277 ± 0.030 and 0.290 ± 0.040 mm wide, whereas the wild group ranges between 0.636 ± 0.039 and 0.665 ± 0.028 mm long by 0.265 ± 0.043 and 0.276 ± 0.029 mm wide. By day 20, these parameters are not significantly different (Tables 4 and 5). As in the earlier days for these flies, histological differences are not identifiable, and three well-defined regions are still observed, each occupying approximately a third of the length of the testis. New spermatogonial cells are added to the growth zone to provide continuity to spermatogenesis. In the middle area, the sperm packed zone continues to undergo cellular differentiation and to release free sperm at the base of the testes, which in some cases occupies up to 40 to 45% of the total length and has an entangled filiform character.

In the group of irradiated males, testicle size continues to decrease due to cell destruction caused by radiation, which completely inhibits mitosis and the differentiation of spermatogonial cells; therefore, there is no possibility of germ cell repopulation, and only the spermatids that were in the process of cell differentiation at the time of irradiation are transformed into dense abnormal sperm. On days 16-20, the free sperm normally occupy the area from the apex to the base of the irradiated testes. The morphometric data of testes exposed to different irradiation doses range between 0.474 ± 0.044 and 0.391 ± 0.031 mm long and 0.183 ± 0.032 and 0.145 ± 0.021 mm wide, with very few significant differences among

them, but with significant differences when compared to the non-irradiated flies. The forms are now more elongated, kidney-shaped and irregular, with the testicular sheath almost entirely separate. These features make the distinction between irradiated and non-irradiated flies relatively easy when captured at this age.

Day 25. (Fig. 20, 21, 25g-I and Tables 4, 5).

At this age the testes of the non-irradiated males do not show any major changes when compared to the previous age group (16-20 days). The testes size of the non-irradiated TSL flies (0.695 ± 0.060 mm long and 0.269 ± 0.029 mm wide) is significantly different from that of wild male testes (0.643 ± 0.043 mm long by 0.273 ± 0.023 mm wide). However, the histological features do not differ, and as in the previous age group, the three regions are perfectly clear and defined and divided into three regions. Occasionally, the basal area becomes slightly larger, which is most likely associated with the ability of different individual flies to transfer sperm. The apical region continues to be intensely stained due to the positive reaction with the aceto-orcein stain. The coloured material of the cell nuclei in the growth zone is well defined, and all of the cells divide evenly to become spermatids in typical cysts or packets. In the sperm packed area, they are abundant; some are compact, and others are weaker and release free mature sperm at the base of the testes that are ready to be transferred to females during copulation.

In the irradiated males, cell destruction continues to be observed, with a consequent reduction in testes size according to the different doses of irradiation: TSL flies irradiated with 100 Gy are 0.395 ± 0.051 mm long and 0.141 ± 0.026 mm wide; TSL flies irradiated with 125 Gy are 0.385 ± 0.047 mm long by 0.135 ± 0.026 mm wide; and TSL flies irradiated with 145 Gy are 0.383 ± 0.038 mm long and 0.142 ± 0.024 mm wide. There are few significant differences among the groups, but if compared with the non-irradiated flies, the difference is obvious. In what was once the growth zone of the irradiated testes, total cellular disorganization is observed with scant remains of very weak bundles, pyknotic cells, few groups of chromatin and very large intercellular spaces caused by dead and disintegrated cells; compact sperm bundles are absent, and the entire testicular cavity is now occupied by dense, abnormal sperm. These testes acquire very thin, kidney-shaped and distorted forms; the testicular sheath or membrane is very separate and in some cases has already disintegrated. Based on these features, the difference with the non-irradiated group is completely clear.

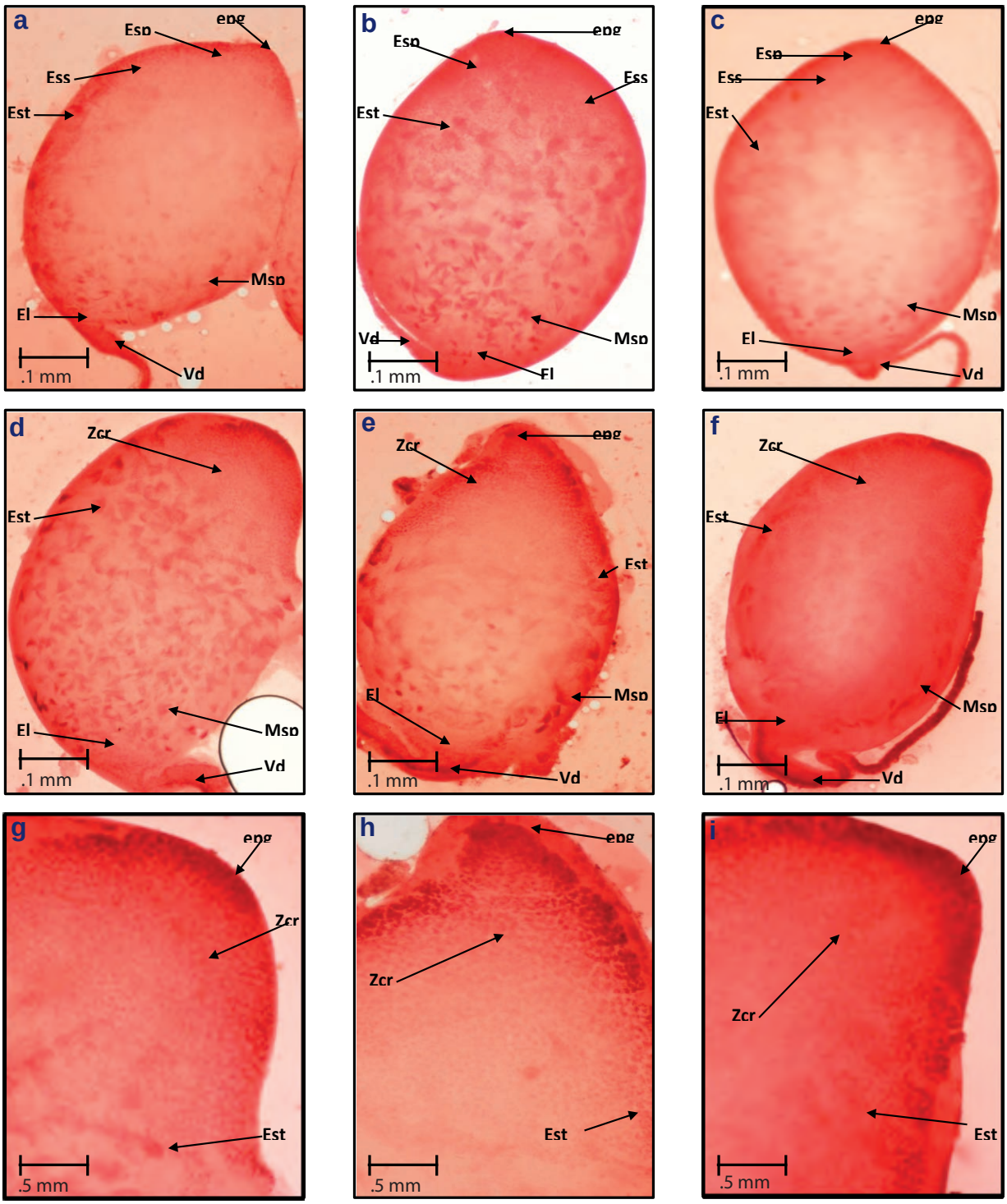

Figure 19. Maturation of testes in non-irradiated TSL males and wild males compared with testes of irradiated males under different treatments: a) Non-irradiated TSL flies at day 0;b) Wild flies at day 0; c) TSL flies irradiated with 100 Gy at day 0; d) Non-irradiated TSL flies at days 1-2; e) Wild flies at days 1-2; f) TSL flies irradiated with 125 Gy at days 1-2; g) Non-irradiated TSL flies at days 1-2, apical region magnified; h) Wild flies at days 1-2, apical region magnified; and i) TSL 125 Gy at days 1-2, apical region magnified: El= Free sperm, Epg= Spermatogonia, Esp= Primary spermatocytes, Ess= Secondary spermatocytes, Msp= Sperm bundles, Vd= Vas deferens, Zcr= Growth area, Est= Spermatids.

Table 4. Testes length from non-irradiated TSL flies and wild and TSL flies irradiated with 100, 125 or 145 Gy (mean ± SD). For each treatment, n = 15 males per day

	Testes length de (mm)									
AGE (DAYS)	NON-IRRADIATED TSL		WILD		TSL 100 Gy		TSL 125 Gy		TSL 145 Gy	
0	0.574 ± 0.058	a	0.466 ± 0.042	b	0.562 ± 0.057	ab	0.551 ± 0.044	ab	0.551 ± 0.037	ab
1	0.576 ± 0.030	a	0.539 ± 0.028	b	0.560 ± 0.038	ab	0.565 ± 0.027	ab	0.555 ± 0.049	ab
2	0.635 ± 0.049	a	0.563 ± 0.043	b	0.567 ± 0.036	b	0.578 ± 0.029	b	0.582 ± 0.027	b
3	0.657 ± 0.036	b	0.570 ± 0.042	c	0.685 ± 0.027	a	0.681 ± 0.030	ab	0.667 ± 0.042	ab
4	0.667 ± 0.029	a	0.587 ± 0.037	b	0.677 ± 0.052	a	0.681 ± 0.049	a	0.683 ± 0.041	a
5	0.665 ± 0.036	a	0.569 ± 0.056	b	0.693 ± 0.028	a	0.687 ± 0.023	a	0.683 ± 0.043	a
6	0.691 ± 0.043	a	0.607 ± 0.052	b	0.591 ± 0.040	bc	0.566 ± 0.067	c	0.575 ± 0.044	bc
7	0.665 ± 0.049	a	0.655 ± 0.061	a	0.576 ± 0.051	b	0.572 ± 0.031	b	0.573 ± 0.030	b
8	0.684 ± 0.033	a	0.636 ± 0.045	b	0.585 ± 0.060	c	0.575 ± 0.030	c	0.568 ± 0.072	c
9	0.689 ± 0.041	a	0.649 ± 0.038	b	0.555 ± 0.019	c	0.517 ± 0.036	d	0.523 ± 0.042	d
10	0.695 ± 0.029	a	0.629 ± 0.033	b	0.525 ± 0.056	c	0.543 ± 0.034	c	0.551 ± 0.033	c
11	0.677 ± 0.023	a	0.637 ± 0.053	b	0.525 ± 0.048	c	0.532 ± 0.045	c	0.518 ± 0.035	c
12	0.671 ± 0.047	a	0.633 ± 0.046	b	0.524 ± 0.051	cd	0.532 ± 0.039	c	0.495 ± 0.043	d
13	0.677 ± 0.051	a	0.621 ± 0.059	b	0.515 ± 0.035	c	0.473 ± 0.042	d	0.509 ± 0.057	cd
14	0.689 ± 0.035	a	0.631 ± 0.043	b	0.511 ± 0.025	c	0.486 ± 0.054	cd	0.479 ± 0.024	d
15	0.689 ± 0.072	a	0.643 ± 0.031	b	0.505 ± 0.054	c	0.489 ± 0.043	cd	0.465 ± 0.029	d
16	0.690 ± 0.060	a	0.636 ± 0.039	b	0.474 ± 0.044	c	0.459 ± 0.038	c	0.457 ± 0.042	c
17	0.695 ± 0.050	a	0.665 ± 0.028	b	0.469 ± 0.031	c	0.456 ± 0.018	c	0.461 ± 0.048	c
18	0.702 ± 0.054	a	0.647 ± 0.075	b	0.397 ± 0.028	d	0.391 ± 0.031	d	0.440 ± 0.068	c
19	0.697 ± 0.045	a	0.651 ± 0.058	b	0.403 ± 0.054	c	0.402 ± 0.059	c	0.416 ± 0.042	c
20	0.699 ± 0.043	a	0.664 ± 0.041	a	0.406 ± 0.063	b	0.393 ± 0.064	b	0.395 ± 0.036	b
25	0.695 ± 0.060	a	0.643 ± 0.043	b	0.395 ± 0.051	c	0.385 ± 0.047	c	0.383 ± 0.038	c

For each value, followed by the same letter in the same row means they are not statistically different (Fisher's exact test, P <0.05).

Table 5. Testes width from non-irradiated TSL flies and wild and TSL flies irradiated with 100, 125 or 145 Gy (mean ± SD). For each treatment, n = 15 males per day.

	With tests de (mm)									
AGE (DAYS)	NON-IRRADIATED TSL		WILD		TSL 100 Gy		TSL 125 Gy		TSL 145 Gy	
0	0.351 ± 0.042	a	0.313 ± 0.038	b	0.333 ± 0.030	ab	0.315 ± 0.039	b	0.339 ± 0.040	ab
1	0.345 ± 0.037	a	0.296 ± 0.030	b	0.293 ± 0.033	b	0.295 ± 0.029	b	0.276 ± 0.036	b
2	0.326 ± 0.036	a	0.305 ± 0.038	a	0.282 ± 0.022	b	0.277 ± 0.027	b	0.276 ± 0.022	b
3	0.326 ± 0.026	a	0.309 ± 0.039	ab	0.292 ± 0.031	bc	0.275 ± 0.019	c	0.276 ± 0.034	c
4	0.323 ± 0.023	a	0.296 ± 0.032	b	0.289 ± 0.033	b	0.289 ± 0.029	b	0.277 ± 0.035	b
5	0.316 ± 0.037	a	0.303 ± 0.043	ab	0.284 ± 0.036	b	0.294 ± 0.030	ab	0.284 ± 0.047	b
6	0.315 ± 0.035	a	0.295 ± 0.034	a	0.236 ± 0.020	b	0.228 ± 0.029	b	0.231 ± 0.026	b
7	0.305 ± 0.036	a	0.295 ± 0.037	a	0.234 ± 0.035	b	0.218 ± 0.020	b	0.223 ± 0.021	b
8	0.295 ± 0.031	a	0.295 ± 0.033	a	0.233 ± 0.039	b	0.223 ± 0.025	b	0.229 ± 0.032	b
9	0.305 ± 0.031	a	0.289 ± 0.030	a	0.205 ± 0.019	b	0.215 ± 0.033	b	0.199 ± 0.025	b
10	0.295 ± 0.030	a	0.285 ± 0.033	a	0.203 ± 0.035	b	0.196 ± 0.029	b	0.209 ± 0.038	b
11	0.295 ± 0.031	a	0.286 ± 0.031	a	0.211 ± 0.043	b	0.198 ± 0.029	b	0.197 ± 0.025	b
12	0.279 ± 0.036	a	0.279 ± 0.023	a	0.185 ± 0.024	b	0.186 ± 0.019	b	0.193 ± 0.032	b
13	0.280 ± 0.033	a	0.284 ± 0.022	a	0.193 ± 0.031	b	0.185 ± 0.023	b	0.185 ± 0.024	b
14	0.287 ± 0.028	a	0.291 ± 0.031	a	0.186 ± 0.031	b	0.187 ± 0.033	b	0.185 ± 0.024	b
15	0.286 ± 0.034	a	0.275 ± 0.028	a	0.185 ± 0.027	b	0.175 ± 0.033	b	0.175 ± 0.027	b
16	0.290 ± 0.040	a	0.270 ± 0.027	a	0.183 ± 0.032	b	0.175 ± 0.019	b	0.165 ± 0.030	b
17	0.285 ± 0.032	a	0.276 ± 0.029	a	0.173 ± 0.032	b	0.165 ± 0.027	b	0.174 ± 0.034	b
18	0.277 ± 0.030	a	0.275 ± 0.032	a	0.175 ± 0.025	b	0.165 ± 0.031	b	0.162 ± 0.026	b
19	0.278 ± 0.030	a	0.265 ± 0.043	a	0.164 ± 0.028	b	0.163 ± 0.028	b	0.151 ± 0.025	b
20	0.278 ± 0.026	a	0.273 ± 0.023	a	0.165 ± 0.023	b	0.145 ± 0.021	c	0.145 ± 0.021	c
25	0.269 ± 0.029	a	0.265 ± 0.024	a	0.141 ± 0.026	b	0.135 ± 0.026	b	0.142 ± 0.024	b

For each value, followed by the same letter in the same row means they are not statistically different (Fisher's exact test, P <0.05).

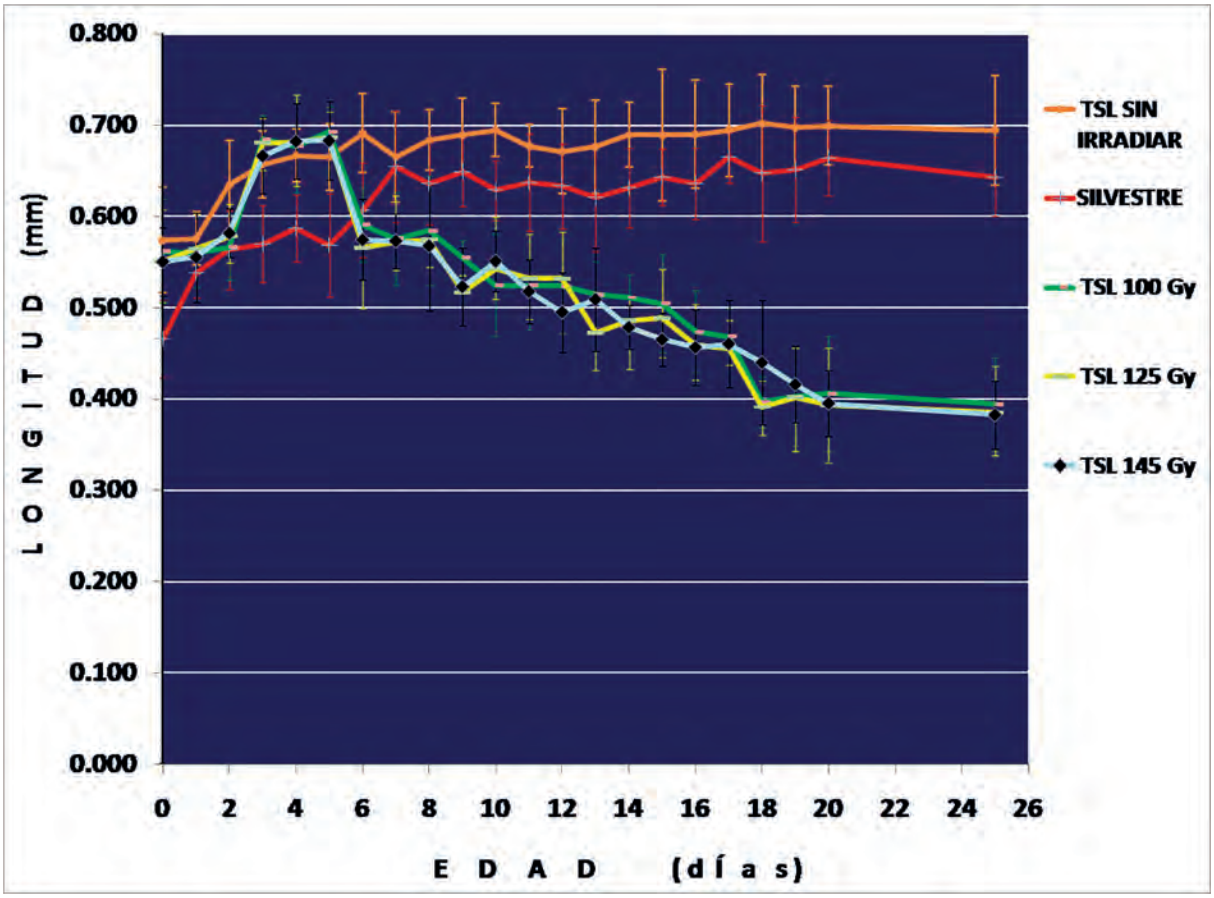

Figure 20. Length of testes of C. Capitata of non-irradiated TSL flies and wild and TSL flies irradiated with 100, 125 or 145 Gy from emergence (day 0) to 25 days old. Vertical bars represent the standard error. For each age n = 15

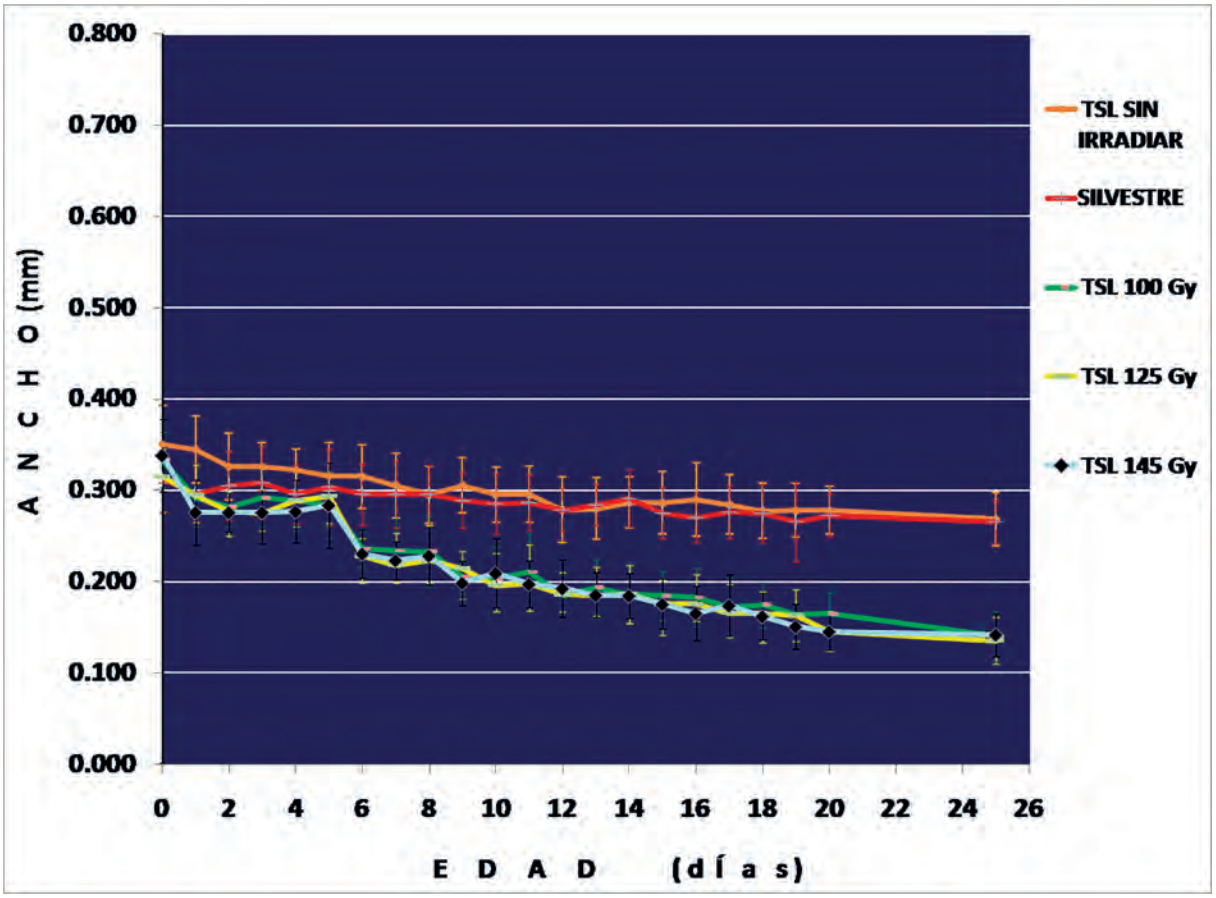

Figure 21. Testes width of Ceratitis capitata non-irradiated TSL flies and wild and TSL flies irradiated with 100, 125 or 145 Gy from fly emergence (day 0) to 25 days of age. Vertical bars represent the standard error. For each age n = 15

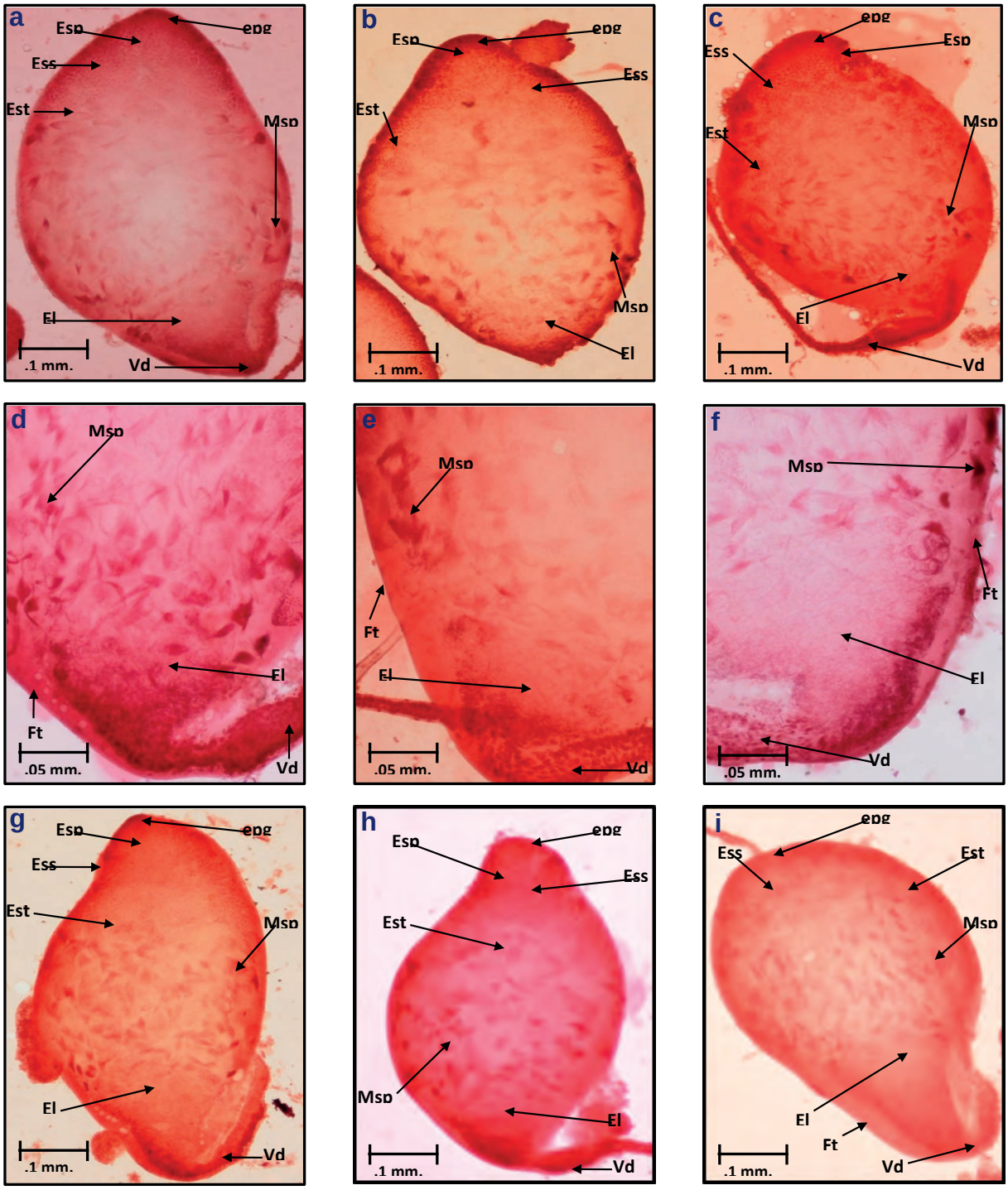

Figure 22.Maturation of testes in non-irradiated TSL flies and wild males compared with the testes of irradiated males from different treatments: a) Non-irradiated TSL flies at days 3-4; b) Wild flies at 3-4 days; c) TSL flies irradiated with 145 Gy at day 0; d) non-irradiated TSL flies at 3-4 days, basal region magnified; e) Wild flies at days 3-4, basal region magnified; f) TSL flies irradiated with 145 Gy at days 3-4, basal region magnified; g) Non-irradiated TSL flies at day 5; and h) Wild flies at day 5; i) TSL flies irradiated with 100 Gy at day 5: El= Free sperm, epg= Spermatogonia, Esp= Primary spermatocytes, Ess= Secondary spermatocytes, Est = Spermatids, Ft= Testicular sheath, Msp= Sperm bundles, Vd= Vas deferens, Vs= Seminal vesicle or base of the testis.

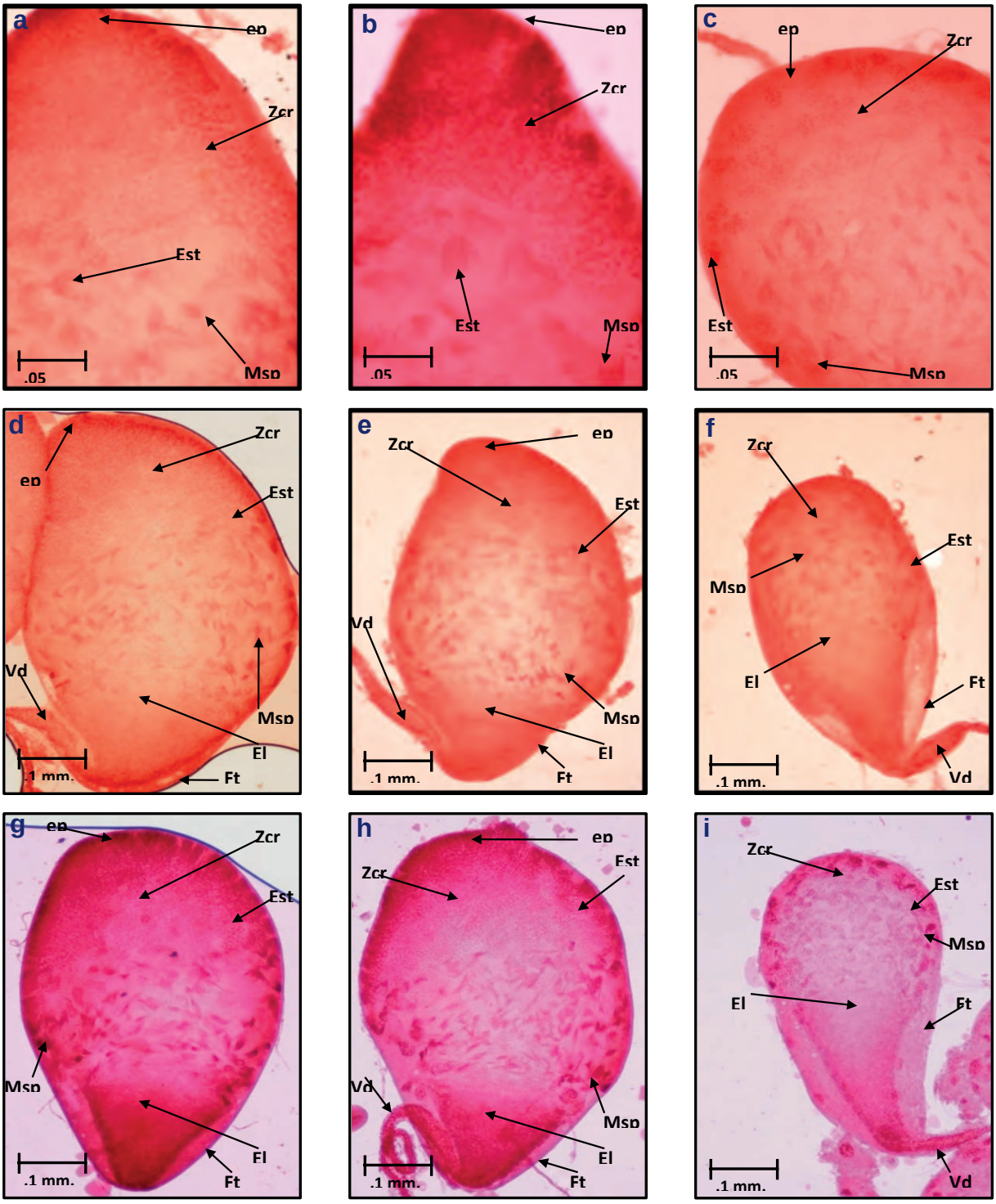

Figure 23.Maturation of testes in non-irradiated TSL and wild males compared with testes of irradiated males from different treatments: a) non-irradiated TSL flies at day 5, apical region magnified; b) Wild flies at day 5, apical region magnified; c) TSL flies treated with 100 Gy at day 5, apical region magnified; d) Non-irradiated TSL flies at day 6; e) Wild flies at day 6; f) TSL flies irradiated with 125 Gy at day 6; g) Non-irradiated TSL flies at day 8; and h) Wild flies at day 8d; i) TSL flies irradiated with 145 Gy at day 8: El= Free sperm, epg= Spermatogonia, Ft= Testicular sheath, Msp= Sperm bundles, Vd= Vas deferens, Zcr= Growth area, Est= Spermatids.

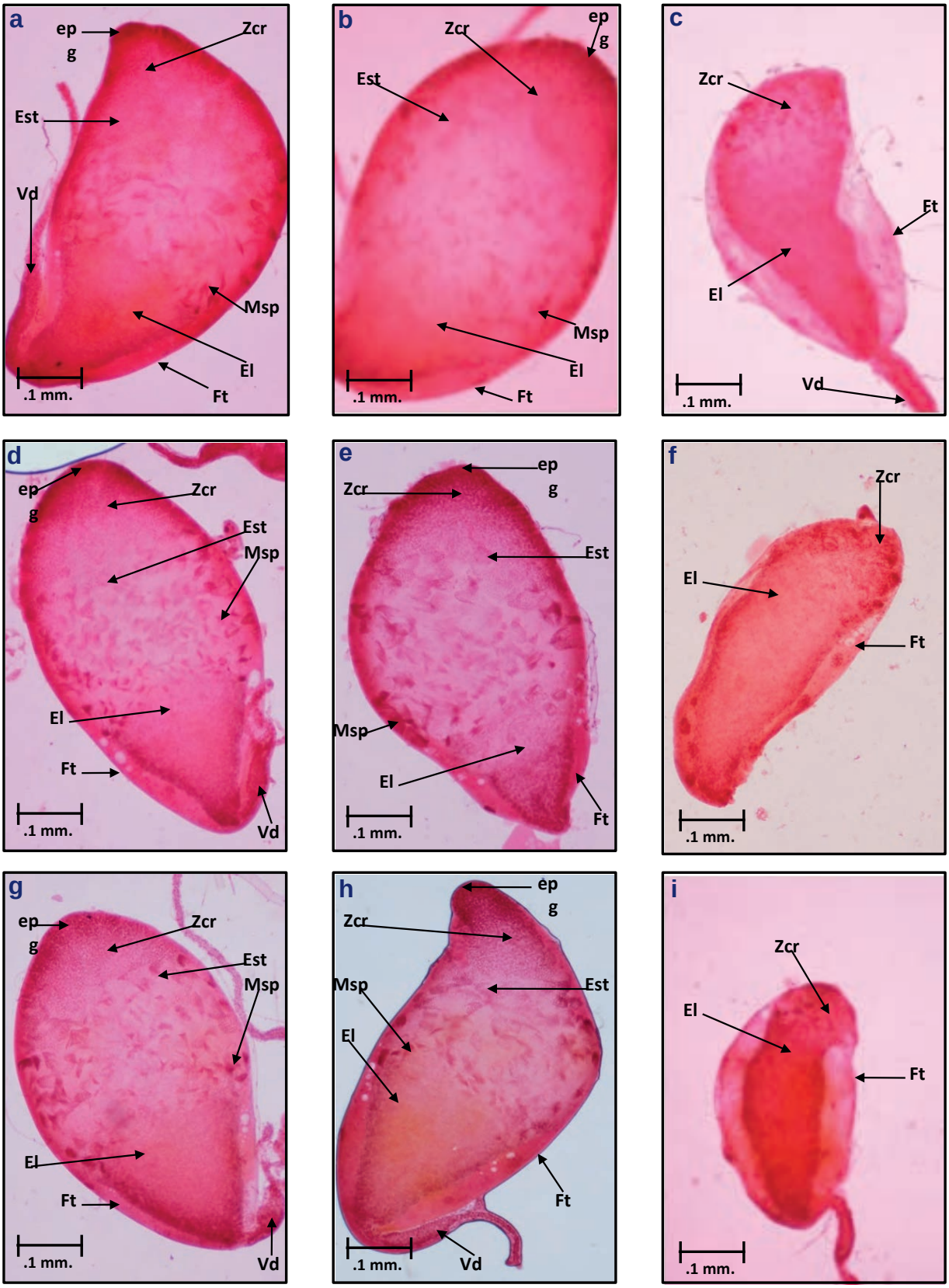

Figure 24.Maturation of testes in non-irradiated TSL flies and wild males compared with testes of irradiated males: a) Non-irradiated TSL flies at day 10; b) Wild flies at day 10; c) TSL flies irradiated with 145 Gy at day 10; d) Non-irradiated TSL flies at day 12; e) Wild flies at day 12; f) TSL flies irradiated with 125 Gy at day 12; g) Non-irradiated TSL flies at day 15; h) Wild flies at day 15; and i) TSL flies irradiated with 145 Gy at day 15: El= Free sperm, epg= Spermatogonia, Ft= Testicular sheath, Msp= Sperm bundles, Vd= Vas deferens, Zcr= Growth area, Est= Spermatids.

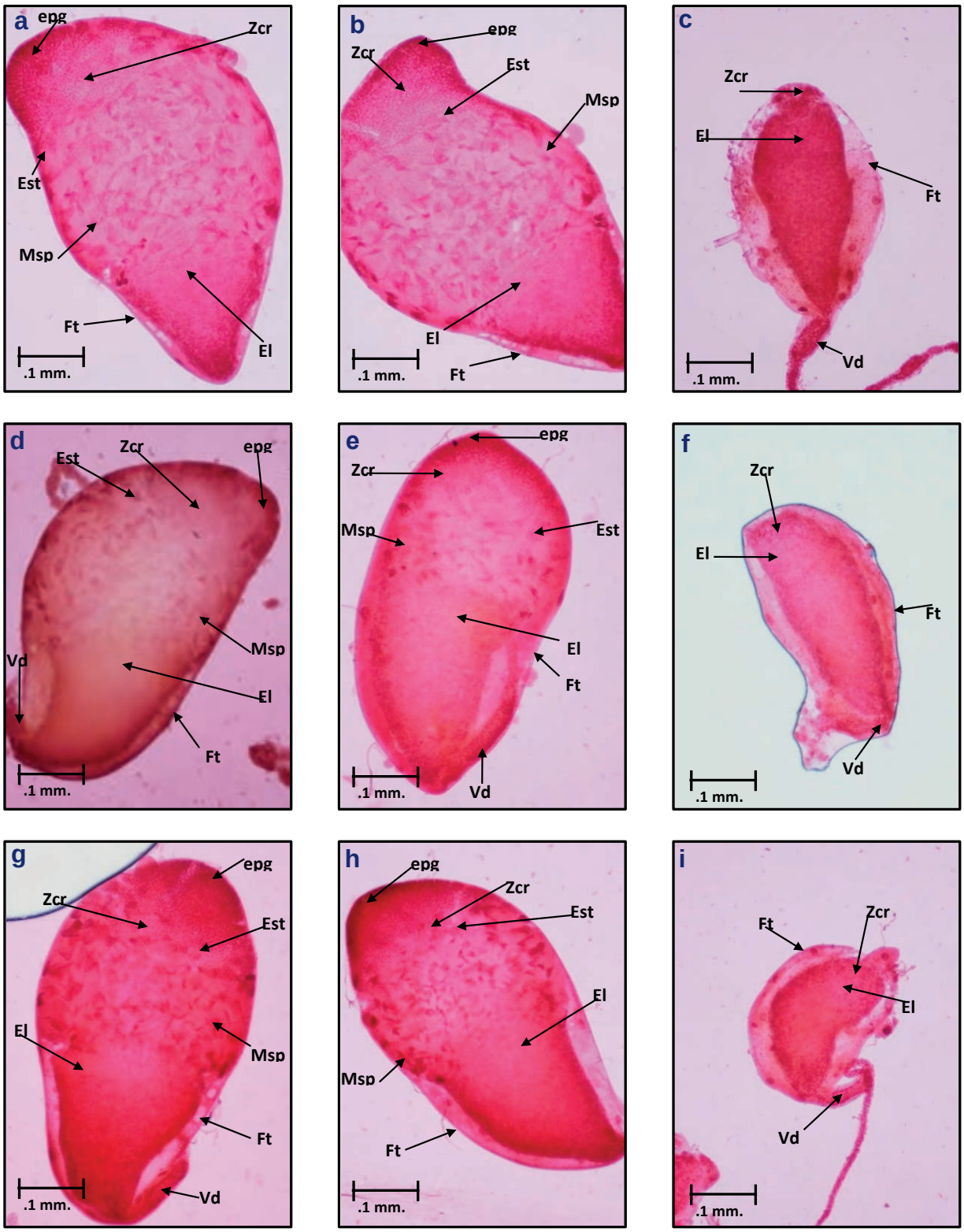

Figure 25.Maturation of testes in non-irradiated TSL and wild males compared with testes of irradiated males: a) Non-irradiated TSL flies at day 17; b) Wild flies at day 17; c) TSL flies irradiated with 100 Gy at day 17; d) Non-irradiated TSL flies at day 20; e) Wild flies at day 20; f) TSL flies irradiated with 125 Gy at day 20; g) Non-irradiated TSL flies at day 25; h) Wild flies at day 25; and i) TSL flies irradiated with 145 Gy at day 25: El= Free sperm, epg= Spermatogonia, Ft= Testicular sheath, Msp= Sperm bundles, Vd= Vas deferens, Zcr= Growth area, Est= Spermatids.

5. RELEVANT LITERATURE

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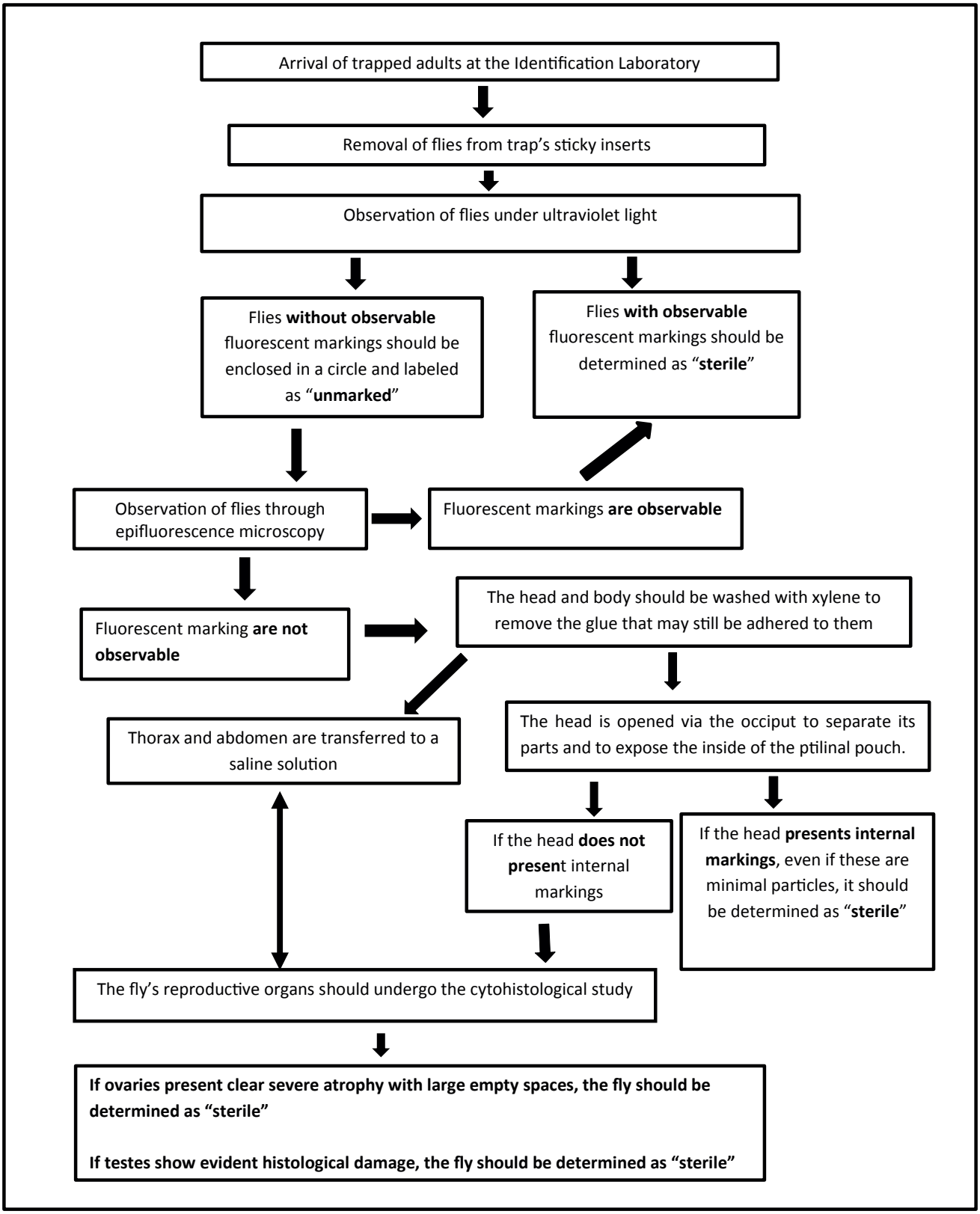
APPENDIX 1

FLOW DIAGRAM FOR DETERMINATION OF MEDFLY STERILE CONDITION



APPENDIX 2

MATERIALS AND EQUIPMENT

Materials

- Dissecting needles (stainless steel)
- Disposable wooden sticks
- Filter paper
- Newsprint
- Stickers (13 x 19 mm)
- Cover plates
- Carrier slides
- Glue (for foil)
- Glass vials, one ounce (30 ml)
- 10-ml glass vials with droppers
- Plastic squirt bottle
- 500-ml beaker (precipitation beaker)
- 10-ml glass pipette

Chemicals

- Xylene
- Mineral oil
- 99% isopropyl alcohol
- Sodium chloride
- Distilled water
- Aceto-orcein
- 90% ethanol
- Mounting medium (Canadian balsam, Entellan or Hoyer liquid)

Equipment

- UV lamp (Black Ray model B-100AP)
- Stereomicroscope with digital camera
- Compound microscope with digital camera
- Epifluorescent microscope
- Entomological tweezers extra fine tip no. 5
- Blunt forceps # 7
- UV protective masks.

APPENDIX 3

PREPARATION OF SALINE SOLUTION

To prepare 500 ml of solution:

Materials

- 6 g of sodium chloride
- 30 ml of 99% isopropyl alcohol
- 464 ml of distilled water
- Beaker of 500 ml
- Graduated glass pipette
- Kitchen scale.

Dissolve 6 grams of salt in 464 ml of water, then 30 ml of alcohol under ambient laboratory conditions.

APPENDIX 4

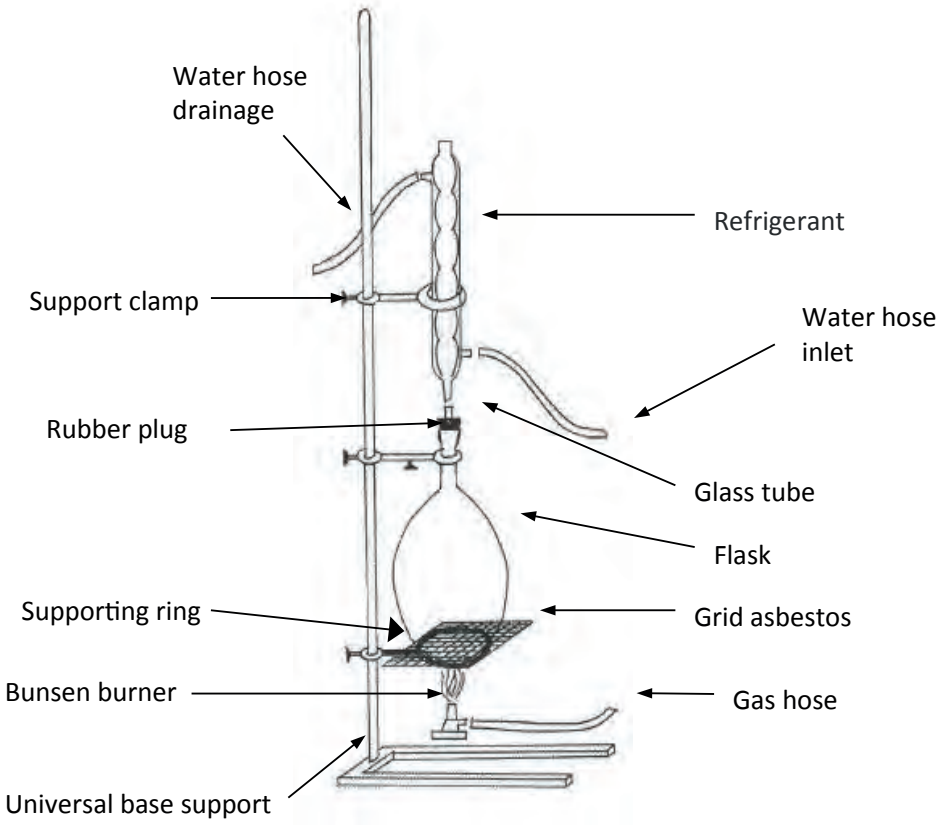
PREPARATION OF ACETO-ORCEIN

To prepare 100 ml of dye:

Materials

- 2 g orcein
- 45 ml of glacial acetic acid
- 53 ml of distilled water
- Filter paper

The equipment required for preparation of aceto-orcein is the following:



The equipment must be set up by placing the refrigerant in the holder and holding it vertically with a clamp and then connecting the coolant to hoses that convey the water. The first hose is placed in the lower projection of the coolant and linked to a water source; in the other projection, the upper hose is the drain. The bottom of the refrigerant must be attached to a glass tube, and the latter is attached by means of a rubber plug or ball to the flask containing the solution.

Two grams of orcein is added to the flask, and then the acetic acid and the distilled water are emptied. These products should be mixed by shaking the flask for one minute.

To homogenize the mixture, it should be boiled for three hours. This solution is then allowed to cool to room temperature, which is then followed by filtration and storage in amber bottles kept in a cool dark place

As a last comment, currently, there are suppliers that already offer aceto-orcein prepared in different formulations (250, 500 and 1000 ml).

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This guideline is an updated version of the one published in 2007. It is aimed at providing harmonized processes involved in the handling and release of sterile insects after production in mass rearing facilities to FAO or International Atomic Energy Agency (IAEA) member countries that want to embark on sterile insect technique (SIT) activities. There is also increased interest by the private sector in investing in sterile insect production and/or other SIT activities, and these harmonized guidelines on the post-production phase will facilitate SIT application and foster the commercialization of the SIT. This guideline resulted from two FAO/IAEA consultants' meetings with representatives of relevant SIT programmes, the first held in Sarasota, Florida, United States of America (April 2004) and the second in Vienna, Austria (August 2005). It also resulted from an in depth review of the first edition, conducted in 2014 and 2015 by SIT program managers and scientists working with SIT technology.

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