Guidelines for Irradiation of Mosquito Pupae in Sterile Insect Technique Programmes

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Cover photo credit: Hanano Yamada

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FOREWORD AND AKNOWLEDGMENTS

This publication is intended as guidance for the irradiation of the pupal stage of *Aedes aegypti*, *Aedes albopictus* and *Anopheles arabiensis*, for routine studies on the biological effects of radiation exposures, in particular, irradiation induced sterility in male (and female) mosquitoes.

The Human Disease Vectors Groups of the Inspect Pest Control Subprogramme, Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture has been investigating the use of nuclear techniques to manage mosquito vectors in a sustainable, environmentally friendly manner, by developing the sterile insect technique (SIT) package for species such as *Ae. aegypti*, *Ae. albopictus* and *An. arabiensis*. The key to this technique is the induction of reproductive sterility in the male mosquitoes which are to be released into the target site where population suppression is intended. Therefore, it is essential to ensure that the methods of the sterilization processes are optimal in inducing the desired effects, whilst minimizing detrimental effects which could decrease the biological quality of the released males.

Following numerous studies on radiation exposures of mosquito pupae, we have found several factors that affect the biological outcome and it is for this reason we decided that a guideline was required to ensure a harmonized approach to the sterilization of mosquito pupae in order to achieve a better and reliable method for the reproducibility of results.

Most of the background information in this guidance is taken directly from the information found in the chapter entitled "Sterilizing Insects with Ionizing Radiation" by Bakri, Mehta and Lance in the book "The Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management", which provides a comprehensive overview of the SIT, and its various components developed over the past 70 years for various insect pests.

The IAEA officer responsible for this publication was Hanano Yamada, under the leadership of Andrew Parker, and Jeremy Bouyer, the current Group Leader of the Human Disease Vector Group, in collaboration with the entire IPCL Team, and we would like to acknowledge and thank the external experts, Romeo Bellini, and Maylen Gómez for their significant contributions to this document. We also would like to thank the Austrian Institute of Technology for the possibility to access and use their Gammacells in addition to ours, on numerous occasions with special thanks to Mr Michael Gems for his time and assistance.

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1. Background information

The basis of the sterile insect Technique (SIT) [1] is the reproductive sterilization of (male) insects and their successful mating with wild females in the wild population. It is therefore essential to standardize methods for the irradiation treatments of the males to reliably achieve the desired sterility, while maintaining health and virility for their mating success once released.

Historically, a variety of chemosterilants were used to sexually sterilize male mosquitoes with varying success and suitability for larger scale SIT programs, and the evaluation of sterilizing male mosquitoes by irradiation has suggested that this is, to date, the most practical, safe and environmentally-friendly way to induce sterility, especially at large scale [2]. The use of isotopic sources for gamma radiation, (usually cobalt-60 or caesium-137) has been most commonly used for area-wide insect pest management (AW-IPM) programmes with an SIT component, however now X-rays and high energy electrons (in this case “high” referring to 1-5MeV) are becoming viable and practical alternatives. In irradiation processes, the key factor is absorbed dose, which needs to be accurately controlled to ensure that treated insects are rendered sufficiently sterile but are still able to compete with wild males and successfully mate with wild females upon release. Therefore, accurate dosimetry (measurement of absorbed dose) is critical. Factors such as insect age and stage, handling methods, oxygen level, ambient temperature, dose-rate and many others prior to and during irradiation, influence both the radio-sensitivity and biological viability of the irradiated mosquito. A careful evaluation of these factors in the design of irradiation protocols can help to find a balance between the sterility and competitiveness of the irradiated males destined for field releases. Many SIT programmes apply higher doses than required as a “precautionary” measure to ensure full sterility, however this is likely to decrease the overall competitiveness of the sterile males which could compromise their effectiveness in the field. Therefore, the studies leading to these guidelines aim to understand the various factors affecting dose-response in mosquitoes in the aim to standardize the irradiation processes to be able to avoid over-dosing and maintain the integrity of the males’ sterility as well as virility.

In living organisms, mitotically active cells, such as stem and germ cells, are the most radio-sensitive cells, and irradiation can make an insect reproductively sterile causing germ-cell chromosome fragmentation (dominant lethal mutations, translocations, and other chromosomal aberrations), that lead to the production of imbalanced gametes and subsequently

the inhibition of mitosis and death of fertilized eggs or embryos [3]. Differentiated cells (somatic cells), i.e. those that no longer divide are generally less sensitive to radiation than stem cells. Therefore, achieving lethality in insects requires a higher radiation dose than achieving sexual sterility. The impact of radiation on somatic cells can be detected by the development of abnormalities, and reductions in longevity, flight ability, mating propensity, and ultimately the death of the insect.

The absorbed dose of radiation is expressed in Système International d'Unités (SI) units as gray (Gy), where 1 Gy is equivalent to 1 joule (J) of absorbed energy in 1 kg of a specified material (1 Gy = 100 rad). If the dose is delivered correctly (and all influential factors are accounted for), efficacy of the irradiation process is guaranteed. Other advantages of using radiation to sterilize insects include: (1) temperature rise during the process is insignificant, (2) sterile insects can be released immediately after processing, (3) irradiation does not add residues that could be harmful to human health or the environment, and (4) radiation can pass through packaging material, allowing insects to be irradiated after having been packaged [4].

Consistent, reproducible and reliable irradiation methods are required to ensure that the target sterility level is reached for millions of male mosquitoes over time, so that no unknown levels of residual fertility can compromise the beneficial effects of the sterile males. It is also essential to balance the high sterility levels targeted, with optimal irradiation and handling protocols in efforts to improve male biological quality to minimize fitness costs and therefore maintain effectiveness in the field. To date, few publications exist reporting the effects of radiation on mosquito sterility and even fewer still adequately describe all parameters controlled for during the irradiation exposures. No two experiments report the same method, nor the same induced sterility (IS) at a given dose for a particular mosquito species, raising the need to standardize procedures and data reporting methods. The Insect Pest Control Laboratory (IPCL) has extensive experience and expertise in the irradiation of agricultural pest species and numerous publications exist describing standardized methods for insect irradiation. This guideline aims to learn from this existing information and utilize relevant components, as well as summarize recent work specific to mosquitoes with the aim of standardizing irradiation procedures for mosquito pupae. All of the irradiation work at the IPCL was performed in Gammacells (GC220) with a Co60 source, or in an X-ray irradiator (RadSource2400).

Gamma-ray irradiators

[*excerpt from Bakri et al., 2005*] "The radiation source consists typically of several source pencils of either cobalt or caesium. The dose rate is predetermined by the current activity of the source, and the operator controls the absorbed dose delivered to the insects by adjusting the time that they are exposed to radiation (an exception — in some large-scale irradiators, several dose rates can be obtained by raising different subsets of the source pencils into the irradiation room). The only variation in the source output is the known reduction in activity (strength) caused by radioactive decay, which can have a significant impact on the programme (financial as well as scheduling) if not taken into account. The activity of a cobalt source, for example, decreases about 12% annually. The irradiator operator compensates for this loss of activity by incrementally increasing irradiation time (approximately 1% each month) to maintain the same predefined dose to the insects. Since irradiation times eventually become impractically long, sources need to be replenished at regular intervals, depending on the initial activity of the source and the operational requirements. Typically, there are two types of gamma irradiators used in programmes that release sterile insects — self-contained dry-storage irradiators, and large-scale panoramic irradiators (Figure 1).

Self-Contained Dry-Storage Irradiators. At present, most sterilization of insects is accomplished using gamma rays from self-contained irradiators. These devices house the radiation source within a protective shield of lead, or other appropriate high-atomic number material, and they usually have a mechanism to rotate or lower the canister of insects from the loading position to the irradiation position. These canisters, which are reusable and generally made of steel, aluminium, or plastic, hold insects during irradiation. To irradiate, a canister is placed in the irradiation chamber while it is in the loading (shielded) position, and the timer is set to deliver the pre-selected dose. On the push of a button, the chamber is automatically moved to the irradiation position. In most self-contained irradiators, the irradiation position is in the center of an annular (circular) array of long parallel pencils that contain the encapsulated radiation source. With this design, the dose is relatively uniform within the irradiation chamber. An alternate method of achieving a relatively uniform dose is to rotate the canister of insects on a turntable. The axis of rotation is parallel to the source pencils, which are usually vertical. The canister stays in the irradiation position for the set time interval, and then automatically returns to the loading position at the end of the treatment. Self-contained dry-storage irradiators provide a high-dose rate but a small irradiation volume (1 to 4 liters) and are suitable for research as well as small-scale programmes that apply the SIT".

Figure 1. Examples of commonly used gamma-ray irradiators. A) Self-contained Gamma Cell 220 with an open sample chamber in the load position, B) a custom-made canister based on stacked petri dishes, and C) a panoramic irradiator where the source is lifted out of a dry pit during irradiation to the center of turntables with samples placed on top.

X-ray irradiators

[*excerpt from Bakri et al., 2005*] "When a beam of electrons strikes material with a high atomic number, e.g. tungsten, X-rays are generated. X-rays, like gamma rays, are electromagnetic radiation. Radiation generated in this manner (by the rapid deceleration of a charged particle) is also known as "Bremsstrahlung" (literally "braking radiation"). While gamma rays from radioisotopes have discrete energies, "Bremsstrahlung" has a broad energy spectrum with a maximum equal to the energy of the incident electrons. Gamma rays from 60Co or 137Cs, and X-rays, penetrate irradiated materials more deeply than electrons. For example, for 60Co gamma rays, dose decreases to half at a depth of about 20 cm in water, but for 10-MeV electrons, the useful depth is only about 4 cm.

The RadSource 2400 (Figure 2), the Wolbaki X-ray irradiator and the Cegelec blood-Xrad irradiator are currently being used in some small-scale SIT pilot projects for mosquitoes. These low energy X-ray irradiators have low penetration, a moderate dose-rate, and thus moderate processing time, but several small containers can be irradiated at a time".

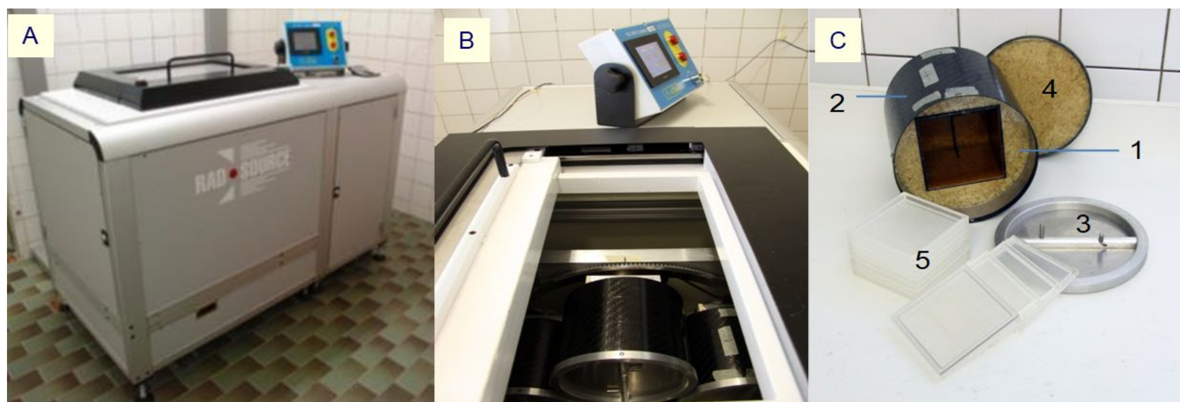

Figure 2. The RadSource 2400. A) the irradiator (cooling system not shown), B) the chamber with 5 rotating canisters with processing capacity of 200,000 Aedes *pupae, and C) a holding canister consisting of 1. Plexiglass cylinder (filled with rice), 2. Carbon fiber canister, 3. Aluminium lid, 4. Plexiglass plug (filled with rice) 5. Stackable plastic plates*

Radiation Dosimetry

[*excerpt from Bakri et al., 2005*] "For the success of a programme using the SIT, the absorbed dose delivered to the insects needs to be accurately quantified and controlled. Also, if contractual arrangements or national regulations prescribe specific doses, the programme will require adequate means to demonstrate compliance. Therefore, the programmes need to have an established dosimetry system to accurately measure absorbed dose and estimate the associated confidence interval, a process known as dosimetry. Dosimetry is performed using dosimeters — devices that, when irradiated, exhibit a quantifiable change in some property, e.g. color, that can be related to the absorbed dose. A dosimetry system includes dosimeters (that are placed into the canister), measuring instruments (to read the change in the dosimeters) along with their associated reference standards, and procedures for using them (ISO/ASTM 2004b). Dosimeters are commonly used in sterile insect production for such tasks as absorbed-dose mapping, process control, and qualification of the irradiator. Several dosimeters are suitable for routine dosimetry at SIT facilities (ISO/ASTM 2004a). Many sterile insect production facilities use radiochromic film systems because they are relatively affordable and are simple to use (IAEA 2004). Procedures for calibrating routine dosimetry systems, and for determining radiation fields in irradiators used for insect sterilization, are described in the International Organization of Standardization/American Society for Testing and Materials (ISO/ASTM) standards (ISO/ASTM 2004a, 2004b, 2004c, 2004d), which are updated periodically, and in IAEA technical reports (IAEA 2002b). Reference standard dosimeters are used to calibrate the routine dosimetry system and radiation fields, e.g. determining the dose rate at a reference position in a self-contained gamma irradiator. Sterile insect production facilities use reference-standard dosimeters for both of these purposes. Externally accredited dosimetry laboratories typically provide these dosimeters and make the readings, resulting in measurements that are "traceable" to national or international standards".

Absorbed-Dose Mapping

[*excerpt from Bakri et al., 2005*] "Ideally, it would be desirable to irradiate all insects in a container (or a canister) at the same dose. In practice, because of the characteristic of radiation interaction with matter, there is a systematic pattern of dose variation within the canister, and therefore not all insects receive the same dose. Dose distribution within the canister is determined by "dose mapping", which typically is conducted by placing several dosimeters at known locations throughout the canister. Dose mapping provides operators of

SIT irradiators with information on the dose within the canister, including areas of maximum and minimum dose, the dose-uniformity ratio - DUR (maximum dose/minimum dose within the irradiation chamber), and areas where the dose rate is relatively uniform (Figure 3). Techniques for dose mapping are described in detail in ISO/ASTM (2004a)".

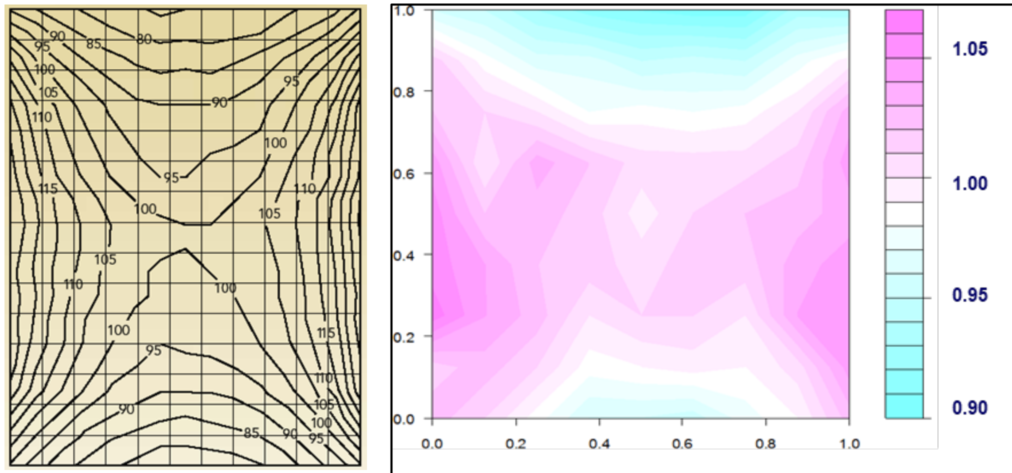

Figure 3. Dose distribution maps. A) a vertical section dose map of a GC220, with doses varying from 75-135% of the center dose (DUR= 1.8), and B) Dose distribution map of a rotating canister in a RadSource 2400 with a -10% and +7% from the center point.

Achieving the desired doses

In radiation studies the primary parameter is dose. For research purposes, it is desirable to achieve doses as close as possible to the target dose with the smallest possible dose variation within the sample being irradiated. Dose rate varies within the available irradiation volume of any irradiator with the distance from the radiation source(s) and the attenuation of the radiation by absorption both in the sample material itself and in the chamber and sample holder material. Therefore, the dose rate must be measured throughout the sample being irradiated (or a suitable dummy material) for each load configuration (load size, shape and position within the radiation field) used to determine if the desired maximum dose variation will be exceeded. The load configuration can then be adjusted to bring the dose variation within the desired limits.

In most Gammacells 220 (Nordion Ltd, Kanata, Ontario, Canada), the dose rate varies substantially throughout the chamber volume, with the lowest dose at the top and bottom of the chamber and the highest at the middle periphery, due to the general positioning of the isotopic pencils (Figure 3). The overall dose uniformity ratio needs to be assessed for each irradiator, but in the GC220, the dose rate varies least in the middle of the chamber. To ensure that no pupae receive less than the target dose, the dose should be measured throughout the sample

volume to determine the point of lowest dose. Once this is known, further exposures can be monitored by measuring the dose only at this point of lowest dose, so long as the sample size and position remains the same. It is equally important that the pupae do not receive more than the maximum acceptable dose, as very high doses will render the resulting sterile males uncompetitive and these will not perform efficiently in the field. When characterizing pupal dose-response, the sample placement should always be consistent in the position for which the dose distribution has been measured. To enable comparisons of the doses applied in different experiments and facilities, a suitable dosimetry system calibrated with traceability to a national standard is required [5]. The calibration provides both a value for the dose received and an associated uncertainty, so that the confidence interval of the measurement can be calculated.

Container shape and material

Container shape and material also should be consistent for experiments and routine irradiation. For Gamma-ray irradiators, the shape of the canister can improve dose uniformity within the canister and thus also the sample by avoiding areas of higher or lower dose rate. For isotopic irradiators, the material of which the canister is made does not affect dose uniformity but will affect dose-rate (depending on the material and thickness of the canister walls). For X-ray irradiators, however, the canister material is important for dose uniformity and dose-rate, as different materials can change the photon spectrum by attenuating low energy photons, which then do not reach the sample. Mass attenuation coefficients for elements and various selected materials can be found on the NIST website (https://www.nist.gov/pml/x-ray-mass-attenuation-coefficients).

Selecting an appropriate dose

[*excerpt from Bakri et al., 2005*] “The absorbed dose that is used to induce sterility is of prime importance to programmes that release sterile insects. As it increases, sterility increases, but insect quality and competitiveness may decrease. Insects that receive too low a dose are not sufficiently sterile, and those that receive too high a dose will be less competitive, reducing the effectiveness of the programme. Quite often, full (100%) sterility may not be the most favourable condition for a programme, and thus process optimization is necessary to balance sterility level and competitiveness, taking into consideration factors that could affect the radiation sensitivity of insects (see later sections) and programme requirements. In reality,

because of the unavoidable dose variability within a canister (as mentioned above), sterile insect production facilities define an acceptable range of doses given to the insects. Most often, programmes or regulatory officials specify a minimum dose that all insects must receive to ensure sufficient sterility. Due to dose variability, most insects actually receive a dose that is somewhat higher than that minimum. An alternate approach is to specify an optimum dose (or central target) and set this as the average or median dose within the irradiated volume of insects. In either case, the DUR should be small; the goal is to sterilize all insects sufficiently without treating large proportions with doses that are high enough to substantially reduce competitiveness. Induced lethal mutations may exert lethality at any stage of development. Quite often, for reasons of simplicity and convenience, the induction of detrimental lethal mutations is made based solely on egg hatchability. However lethal mutations occur at all developmental stages. Therefore, researchers should measure dose effects all along this developmental continuum, or the actual survivorship from egg to adult, to give a true picture of induced sterility. As a result, 99 or 100% sterility in the egg stage is not essential, nor desirable, if it drastically reduces the competitiveness and vigor of the sterile insect. An informed decision on treatment dose requires accurate data on how factors such as dose, insect stage and age, and various process parameters affect levels of sterility and insect quality. For programmes that apply the SIT, the accuracy and value of such data depend on the use of standardized dosimetry systems, procedures, and reporting methods (ISO/ASTM 2004c). Published data on the radiation biology of the same or similar species can provide guidance, but, in many cases, are of limited value because dosimetry procedures, dose-measurement traceability, dose distribution, and other pertinent information are often not reported. In addition, the details of insect-handling procedures, and, perhaps, strain-related differences, can influence radiation sensitivity (see later section on factors that affect dose-response)".

To determine the optimal dose for mosquitoes, it is essential to establish a dose-response curve for each strain, and for each individual situation (including the standard rearing methods, and the available irradiation device(s)). The methodology is discussed in later sections.

2. Irradiating mosquitoes at pupal stage

Selecting pupal age

In general, later developmental stages are more resistant to radiation; i.e. larvae are more susceptible than pupae, and these are more susceptible to adults. Similarly, it has been demonstrated in mosquitoes and other insects that radioresistance increases with pupal age [6–8]. It is therefore important to set the fully sterilizing dose for the oldest pupae present in the sample, and for sample ages to remain consistent for experimental work or routine irradiation events.

In order to account for pupae age, optimal and synchronized larval rearing greatly enhances the efficiency of obtaining pupae of the same age. Ideally, a large proportion of the larvae pupate on a known day post egg hatch: for *Ae. aegypti* and *Ae. albopictus*, this is often on day 5 post hatch, and for *An. arabiensis*, day 7 post hatch (which of course can vary depending on rearing methods) [9]. When first pupae appear in the larval rearing trays, these should be removed, and the time recorded. Then all pupae that form over a 4 hr (or 6 hrs, etc) window can be collected again and the age range of the sample is known. Ideally, the time window should be kept short, as the smaller the age range, the more uniform the induced sterility in the individuals following radiation exposure. The collected pupae of known age can now be kept for a predetermined number of hours to ensure the irradiation occurs at the desired age. For example, pupae are collected in a 4-hr window, and they are irradiated 40 hrs after collection, so the age range of the pupae will be 40-44 hrs (see the protocol for establishing the dose-response curve for *Aedes* spp, Annex I). It must also be taken into account, how long the overall pupal duration is of a given mosquito strain, and irradiation duration that is needed for a given irradiator, to avoid emergence of adults during irradiation.

Preparing pupae for irradiation

Once pupae have been collected and sexed either using the glass pupal sorters [10], the sieving method [11], or by visually separating pupae based on the genitalia using a microscope [12], the pupae can be counted into batches of equal numbers into small containers using a pipette. A 3 ml plastic pipette is generally large enough to collect the pupae, however the tip may be trimmed with a scissor to ensure that the pupae are not damaged by getting stuck in the pipette tip. It is important that the pupae are not subjected to unnecessary stress, or that different

groups receive different treatments or stress factors, as this may change their responses to irradiation and present with varying results.

Consistency in environment, materials and methods

Container. As discussed in the section above "Container shape and material", these attributes are important for consistency in irradiation procedures. For example, the GC220 chamber is cylindrical, therefore the canister for irradiation should also take this shape (example shown in Figure 1B, Figure 4A-C). The container does not need to take up the entire space in the irradiation chamber, and irradiation work can be done in smaller container, but these should be placed in the center of the chamber. The smaller the area of sample placement, the better the dose uniformity among the sample.

Various materials can be suitable for the construction of a canister, as long as the attenuation coefficient is taken into account. For example, polymethylmethacrylate (PMMA) (can be replaced by Styrofoam, however the thickness of the canister walls would need to be increased as the density of the material is lower. More information regarding materials and their attenuation can be found in the next section "Build-up material".

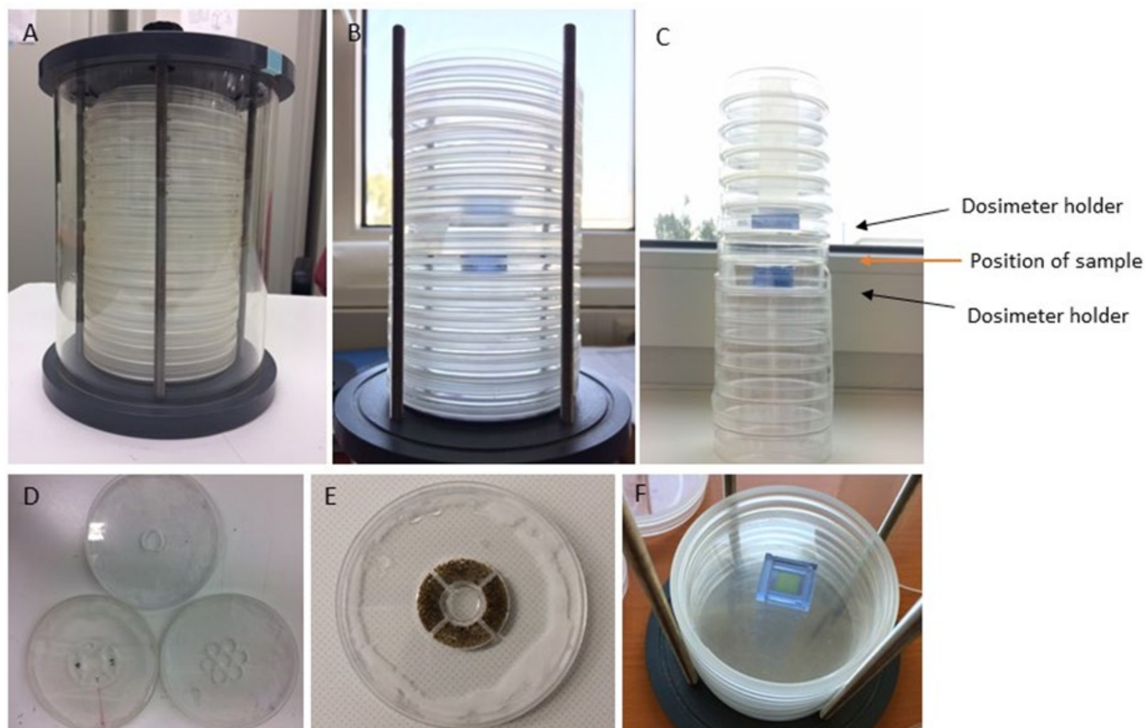

Figure 4. Holding containers for the standard irradiation of mosquito pupae at laboratory scale. A) custom made canister made of stacked petri dish (without lids) held together by plastic rings, surrounded by a Plexiglass tube to provide adequate build-up material. B) Central position of the sample and dosimeter holders (plastic box) in the canister (A). C). Petri dishes (60 mm x 12 mm) stacked and taped, with central position of the sample and location of dosimeters. D & E) Custom made inlays for standard petri dishes, with holes for positioning of pupae samples at equal distances from the center and edges, for uniform absorption of dose. F) Plastic dosimeter holders containing three (1 cm X 1 cm) Gafchromic films.

Build-up Material. Build-up material is very important for the standardized and uniform irradiation of samples, because it generates a standardized electron field (Figure 4). There are two competing effects that stabilize the electron field within the sample container and sample itself the generation of high energy electrons dislodged from the material by the incident photons and the decay of the high energy electrons as they interact with the material; the high energy electrons can ionize further atoms in the material, transferring part of their energy to the new electron so that there are progressively more free electrons at progressively lower energies, until the energy of the electrons falls below the ionization energy of the material and no further free electrons are released . It is these many low energy electrons, while they still have enough energy to ionize the molecules in the insect tissue, that cause the dominant lethal mutations and somatic damage, and which are measured by dosimeters.

The distance through the material for a high energy electron to decay to many non-ionizing electrons depends on the initial energy of the electron, the atomic composition of the material and its density. Energetic photons only interact rarely with matter, so near the surface of the material few high energy electrons are released, and progressively more are released further into the material (Figure 5A). At the same time, these high energy electrons are decaying, and the two processes reach an equilibrium at a characteristic distance into the material, the electron equilibration distance. If the material is too thin the equilibrium is not achieved and the electron field will continue to build up inside the sample, giving a rapidly changing dose rate in the first few millimeters of the sample. If it is thicker than the equilibrium distance it will attenuate the photon beam somewhat (i.e. reduce the dose rate).

For the irradiation of mosquito pupae with ^{60}Co, a 4mm thick PMMA layer is needed. PMMA (Plexiglass, Acrylite/acrylic glass, Perspex or Lucite) is recommended, as its interaction with ionizing radiation is near equivalent to water (as are pupae, and biological tissue in general). For X-ray irradiators, this configuration should be kept for standardization, even though the build-up material in this case is more than necessary (at 150 keV, 100 microns is sufficient), and will decrease the dose-rate. Thus, dose-rate should be measured inside the container for calibration and calculation of dose-time.

The absence of build-up material will affect the actual dose received by individual pupae in the sample, as pupae located at the edge of the sample (Figure 5) will get less effective dose than those in the center of the sample, for which the build-up of electrons is sufficient, thus delivering the full target dose.

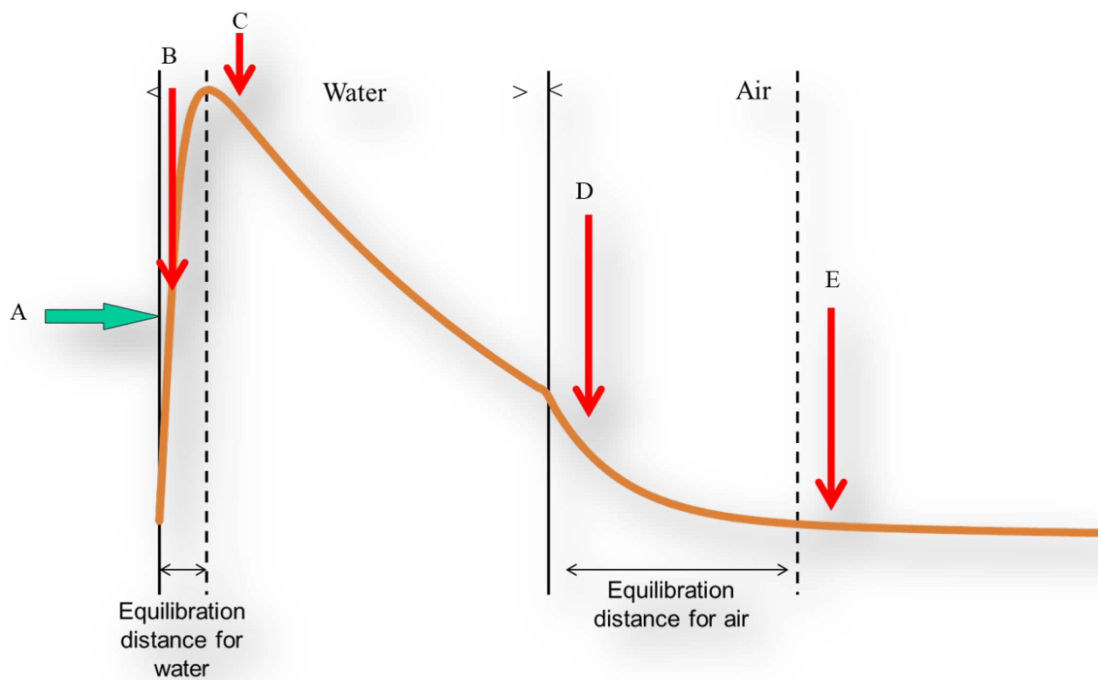

Figure 5. The importance of build-up material in irradiation. Canister material (and its density) and wall thickness are important factors.

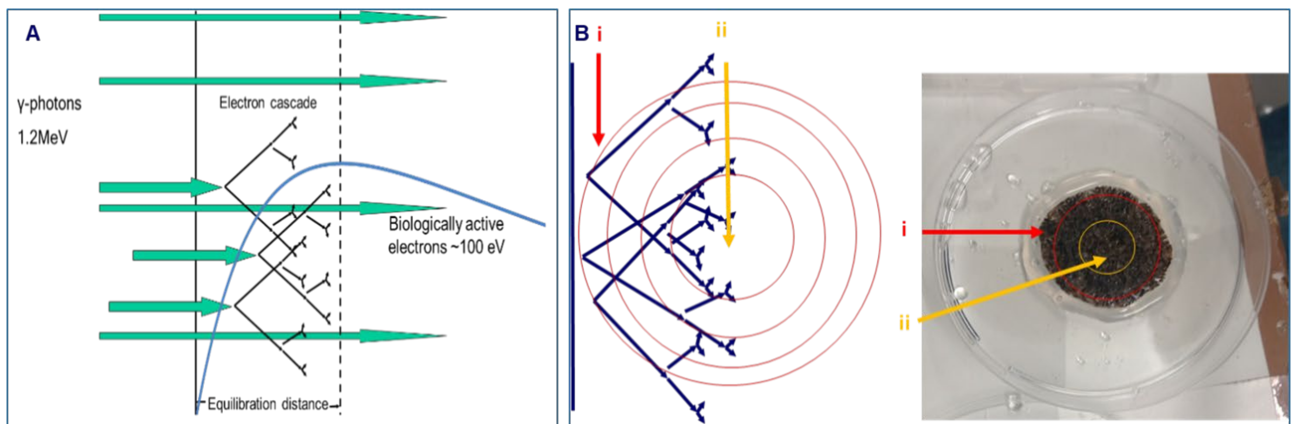

Figure 6. The equilibrium distance and electron cascade in exposed samples. A) graphical presentation of the electron equilibrium in the presence of build-up material. B) Effective dose received by a monolayer of pupae positioned in the center of a petri dish in the absence of surrounding build-up material. B.i) outer layer of pupae receive less effective dose, but serve as build-up material for inner laying pupae, which receive more dose (B.ii).

Positioning the pupae. When the collection, sexing and quantification of the pupae is complete, they are ready to be transferred to the irradiation canister/container. Generally, standard 100 mm x 15 mm, (or 60 mm x 12 mm) Petri dishes are suitable and readily available in any laboratory. These can be either stacked and held in place in a custom-made canister simply made with plexiglass (PMMA) and some screws (Figure 4) or if this is not available, then the lids can be added and the petri dishes stacked (Figure 4C) and held in place with a PMMA tube (ideally 4mm) which will also serve as adequate build-up material. It is important

that the pupae are placed in the center, or equidistant from the center /edges of the Petri dishes (Figure 4E). This can be facilitated by either making special Plexiglas inserts for the Petri dishes (Figure 4D) or more simply, to make a ring out of hot-melt adhesive in the centre of the plate (Figure 6B).

Pupae densities. For routine irradiation at experimental scale, pupae should be placed in a monolayer, with excess water removed by pipetting so that pupae are damp, but not swimming around or submerged under water. Therefore, the container size should accommodate pupae densities, and these should be kept the same for replications or standard experiments assessing dose-response. More pupae will cover a larger area in the petri dish, and therefore there will be an increased variation in absorbed dose amongst individual pupae.

Ambient temperature. The extent to which ambient temperature affects the dose-response in mosquito pupa has not yet been thoroughly investigated. However, it has been shown that temperature impacts metabolic rates, and therefore may affect cellular responses to radiation exposure. Therefore, it is important to keep the temperature of the environment the same between replicates and experiments during irradiation. At the IPCL, the temperature for irradiation is maintained between 20-25˚C. The temperature within the canister should be measured in any case, as temperature affects the development of dosimetric films and the temperature values are required for the reading and analysis of the films the following day post-irradiation.

Routine dosimetry

A dosimetry system is required to verify the dose received by the batches of pupae. Generally, a system based on Gafchromic HD-V2 and MD-V3 film (Ashland Advanced Materials, Bridgewater NJ, USA) is easy to use and adequate for laboratory experiments in irradiation. Firstly, the films need to be calibrated using the irradiator that will be used for the experiments. HD-V2 films are suitable for a dose range of around 20-1000 Gy, whereas MD-V3 film is more suitable for doses of 70 Gy and lower. MD film has protective layers on both sides of the active layer, which makes is relatively resistant to water. However, the HD films have a protective layer only on one side and should therefore not get exposed to water. Both types of films can and should be protected by placing them into sealed plastic, or aluminium envelops, or paper envelopes if not in contact with water (Figure 7B and C), which can be stuck to the top and bottoms of the Petri dishes, on either side of the samples. Note that dosimetric films also require adequate build-up material. For exposures where this is not already available,

small PMMA dosimeter holders can be made or bought (Figure 7 7B, right). Following the radiation exposure, the dosimetry films should be read the next day at the same time, i.e. after 24 h, with an optical density reader (Figure 7A).

A comprehensive guideline on the Gafchromic system is available on the IAEA website, which also discusses other dosimetry systems as alternative options [5].

Figure 7. Gafchromic dosimetry system. A) the optical density film reader, B) paper envelope containing 3 exposed films (left), and a plastic dosimeter holder containing 1 unexposed film, and C) aluminium pouches that can be heat sealed, with an adhesive strip for sticking the envelope onto a container.

Adult emergence

Once the pupae have been irradiated, they are returned to the insectary where they are allowed to emerge in separate cages . Adult emergence rates should be recorded as one of the QC parameters for irradiated mosquitoes. Pupae irradiated at older ages are less prone to succumb to the handling and irradiation treatments. Mortality rates in pupae batches should not exceed around 3% when irradiated at ages older than 36 h, and at doses inducing around 99.0% sterility. More than 3% failure to emerge indicates problems in either handling methods or irradiation itself, and protocols, and actual dose received (dosimetry) should be re-checked.

Assessing male sterility

Once the irradiated males have emerged in their respective cages, the cages should be checked for the presence of any females which may have passed undetected through the sexing procedure. These should be removed, as their induced sterility is likely to differ from their male

counterparts and will skew the sterility data. When males are ready to mate (2-3 days post-emergence), virgin females of the same strain are added to the cages at a 1:1 ratio and are allowed to mate for 2-3 days to ensure that all females are inseminated. Generally, 2 days are sufficient. Three days are suggested to accommodate the weekends as planned in the schedule provided in the protocol for establishing the dose-response curve for *Aedes* spp, (Annex I). After the mating period, the females are offered a bloodmeal, preferably on 2 consecutive days (to ensure that most, to all females bloodfeed). Egg cups with oviposition papers are then provided in each cage for oviposition. Two-three days are allowed for all females to deposit as many eggs as possible. For *Ae. albopictus* and *Ae. aegypti*, the egg cups are collected, and the water carefully removed from the cups, while keeping all of the eggs (including any loose eggs) in the cups. The cups containing the oviposition paper with eggs, and loose eggs at the bottom of the cup can then be slowly dried over 3 days before letting them dry completely before hatching after, for instance, 14 days, as described in the guidelines for routine colony maintenance of *Aedes* spp. [13]. Other research groups and publications suggest hatching before 14 days. Egg hatching protocols should therefore be tested and optimized for each strain and insectary condition.

For *An. arabiensis*, egg cups are collected containing the wet filter paper and eggs hatched on the same day as described in the guidelines for the standardized mass rearing *Anopheles* mosquitoes [9].

Egg hatching should be allowed for a period of 2 days to allow time for “late hatchers”. Then all L_1 larvae are counted and removed, before egg hatch rates are counted and verified under the stereomicroscope. It is often difficult to properly see the status of the eggs. Hatched eggs can be determined by observing the missing tips of the eggs (Figure 8B). However, it is difficult to see this when eggs are rotated in an angle where this missing egg cap is not obvious. Eggs that look unhatched should be rotated with a dissection needle to verify the hatch status. Unfertilized (sterile) eggs may also appear to be unhatched (Figure 8C) or deflated. These can also burst which make them have the appearance of a staple (Figure 8A). These eggs should be counted as “unhatched” or “sterile”. The proportion of these burst eggs tends to increase with increased dose, and therefore sterility. It is essential to account for all of these eggs in the final determination of induced sterility.

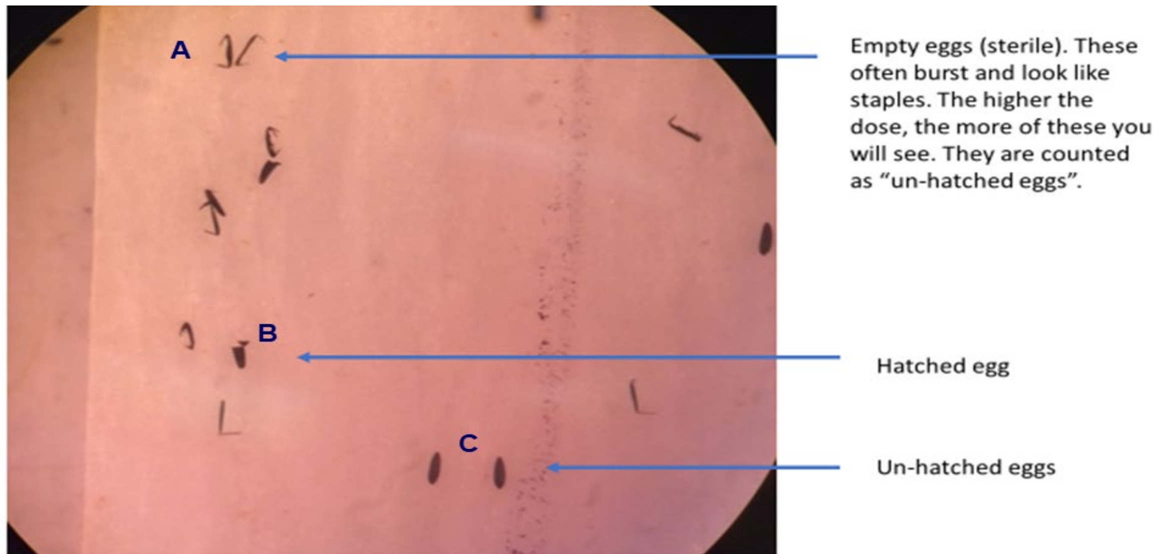

Figure 8. Counting hatched and un-hatched eggs under the stereomicroscope. A) unfertilized, empty eggs that have burst, having an appearance like a staple, B) hatched eggs with detached egg cap, and C) intakt, unhatched eggs, which may or may not contain an embryo.

The eggs that appear to be unhatched can have one of two statuses: they can be simply unhatched, but fertilized, i.e. containing an embryo, or they can be unfertilized, i.e. empty and still intact. This can be elicited by either bursting the egg with a dissection needle (which will either burst the empty egg (Figure 9A-D), or will release the yolk and embryo), or by bleaching the remaining eggs for ca. 10 minutes in a 6% sodium hypochlorite solution. The bleach will dissolve and remove the egg chorion and will expose any unhatched embryos. The embryos are hard to see as they are very small and essentially transparent, however they can be identified by the presence of the 2 eye spots (Figure 9E and F). The number of embryos should be recorded in addition to the number of L1, hatched, und unhatched eggs. If the number of eggs laid are low (less than 100 eggs), suggesting that only one, or very few of the females laid eggs, this may not be representative of the induced sterility of the male population in a particular batch. The more females that lay eggs, the sounder the data. It is possible to offer additional bloodmeals and then collect the second batch of eggs and combine the data in the end.

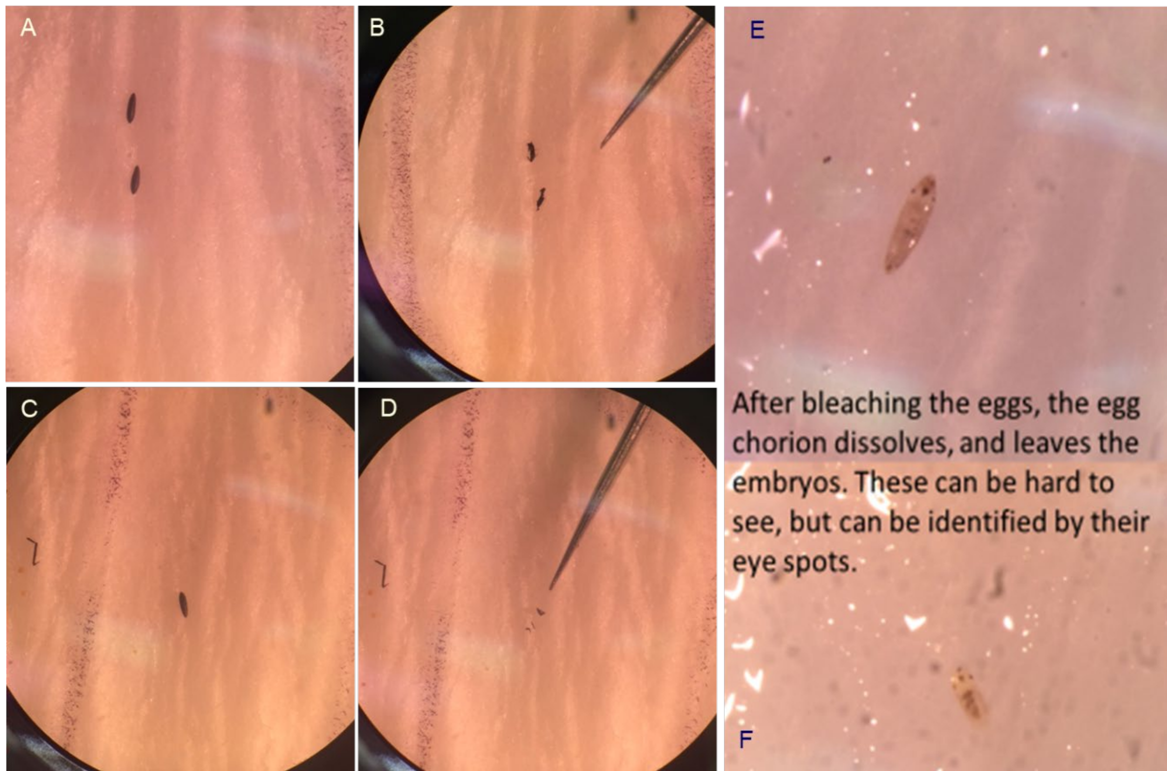

Figure 9. Determining fertility status of unhatched eggs. A) eggs appear to be unhatched and intact. B) the same eggs after being burst with a dissection needle. C) Intact egg. D) egg after burst with a dissection needle, giving an appearance of a staple. A-D) these intact eggs are all empty, and therefore unfertilized/sterile. They are counted as "un-hatched" or "sterile" when computing sterility/fertility rates. E&F) Intact eggs that have been bleached. The egg chorion has dissolved completely, exposing the embryo. These can be seen by the 2eye spots. The embryos are counted as "Hatched" or "Fertile" when computing sterility/fertility rates.

In addition to this basic data, it is useful to follow any surviving larvae to adulthood. Lethal mutations can induce mortality essentially at any life stage. So, although induced sterility after irradiation is often computed by simply relying on hatch rates, or the presence or absence of an embryo, the true overall effects of the radiation exposure can only be verified by following any survivors through all developmental stages until the adult stage. It may not be necessary to apply a high dose such as to reduce egg hatch to >99.9% if most or all of the surviving embryos die along their development before reaching adulthood. This way, it may be possible to reduce the total dose given, and thereby reduce unnecessary reductions in other biological qualitative parameters. The data will later contribute to the final analysis of results, depending on what information is desired. Combining all of the information, including each data types' uncertainties, will provide an overall, and clearer picture of the dose-response in a given mosquito strain in a particular setting.

- Hatch rate: which is the proportion of eggs hatched from all eggs (no. of hatched eggs/total no. of eggs). Uncertainty: it is not clear whether eggs that appear unhatched are sterile, or fertile and has simply not hatched for whatever reason.
- Viable L_1: is the proportion of eggs that resulted in viable L_1 larvae. (no. L_1/total no. of eggs). Uncertainty: it is not clear if any L_1 died, or will die shortly after hatching, or whether all fertile eggs indeed hatched, and whether larval mortality was caused by the irradiation or other external factors.
- Fertile eggs: is the proportion of eggs that were successfully fertilized by fertile sperm (no. of L_1 + no. of embryos/total no. of eggs), or inversely, Sterile eggs: is the number of unhatched, unfertilized, empty eggs over the total eggs. Uncertainty: it is not clear whether the embryos that failed to hatch never were going to hatch due to mutations that inhibited hatching or viability, or whether they did not hatch due to external factors.
- Viable adults: represents the eggs that were fertilized by fertile sperm, with no lethal mutations, allowing for fully viable, and potentially reproducing offspring resulting after irradiation of the parent. This data gives a better picture of what total effects the irradiation treatments induced, and whether the dose needs to be increased or decreased. The presence of hatched eggs, and larvae are not significant if these never successfully reach adulthood. Uncertainty: a good untreated control is required to correct for natural mortality.

Assessing female sterility

Female insects have been shown to be more susceptible than conspecific males to irradiation, meaning that they generally require less dose to induce full sterility. However, in some insect species, if female pupae are irradiated too close to emergence, they may have already developed some oocytes that can become viable eggs even after radiation exposure [14]. It is therefore again, important to control the age of the female pupae when planning irradiation experiments.

To assess induced sterility in irradiated females, these are mated to fertile males when both sexes are sexually mature, (preferably virgin males to ensure insemination capacity), for a 3-day period before being offered bloodmeals. Again, 2 bloodmeals on consecutive days are recommended to ensure that all females have had an adequate intake of blood for potential egg production. After oviposition (if any), data on fecundity, hatch rates, viable L_1, number of unhatched embryos, and the adult emergence rates should be recorded as described in the previous section.

3. Establishing a dose-response curve

Selecting appropriate doses

For choosing appropriate doses for establishing a dose-response curve for a given strain and irradiation device, it is recommended to select a series of (more than three) doses in a geometric sequence, so that when the response (induced sterility) is plotted against (log) dose, the points are equally spaced. The dose ranges can be selected based on past studies and data found in the literature that induce sterility rates in the range of 50% - 99%. For *Ae. aegypti* and *Ae. albopictus*, these are 20, 55, 70, 90 and 110 Gy; and for *An. arabiensis*, these are 40, 75, 90, 110, 120, for a GC220 with a dose-rate >80 Gy/min., and in normoxic conditions [2,15].

True replicates and pseudo-replicates

True replicates for irradiation studies involve the repeat of the experiment at a different time, and using a different cohort, comprising a separate irradiation event altogether. True replicates of eliciting dose-response provide a more realistic picture of the possible variations in effects, or cohorts of the same strain. The more of true replicates, the more reliable is the information, and the more confidence can be given to the methodology and results.

Pseudo- replicates in this case are useful to give data for each true repetition statistical strength, and to provide a “safety-net” for possible mistakes or external factors, for example:

Simple unfortunate events can happen in one or more of the replicates, such as individual mosquitoes escaping, or dying. Some cages may contain males that didn’t mate, or females that fed poorly on the bloodmeal (either because of some external factor (ex. position of the cage, such as next to an air vent, or cold source that may change the behavior of the mosquitoes), or quality of blood in one of the bloodfeeders, etc.). Changing the number of individuals, or sex ratios in a cage may result in less females laying eggs, and different total egg numbers produced, which can affect the final hatch rates as these may represent only a few individuals’ induced sterility level and not the accurate average of the whole male population in that particular cage. Mistakes can happen. It is easy to miss a male during the sexing of virgin females, and a single male in your stock of virgin females, can ruin not only a repetition, but the entire experiment. Therefore, it is wise to have multiple checkpoints in your sexing of virgin females. First at pupal sexing stage, then again after emergence in individual tubes at adult stage, and then again once you have transferred the females from the tubes into cages for

their keeping until the mating crosses can be done for the experiment. It is also wise to keep virgin females in as many cages as there are reps, and add females from cage 1, to the rep 1s, and females from cage 2, to the rep twos, and so on. This way, if there is a female cage that did contain a fertile male, this can be accounted for throughout all of the reps with females coming from that particular cage. It could be that one fertile male makes it into one of the experiment cages, completely skewing the sterility level in that repetition. These incidences are quite obvious, and if this is suspected, the results from that particular rep can be explained and censored in the data. Hatching can also be tricky for *Aedes* spp. It is good to have several replicates of the egg hatching for each treatment (dose). Embryo maturation, storage methods and hatching might be slightly different in different egg batches, so the more reps that are available, the better the information acquired in the end. It is therefore essential to have several true replicates for the treatment you are testing (i.e. irradiation doses, methods, etc.) and also several pseudo-replicates for the mating, egg production, egg hatch, longevity, etc., for each true-rep that is done.

Protocol for the establishment of a dose response curve for Aedes albopictus and Ae aegypti (Appendix I)

Protocol for the establishment of a dose response curve for Anopheles arabiensis (Appendix II)

4. Factors that can affect the dose-response in mosquito pupae irradiation

Biological factors

General

[*excerpt from Bakri et al., 2005*] "The most radiosensitive cells are those (1) with a high mitotic rate, (2) with a long mitotic future (i.e. under normal circumstances, they will undergo many divisions), and (3) which are of a primitive type. These generalizations, with some exceptions, have become known as the Law of Bergonie and Tribondeau. In this regard, germ cells are the most radiosensitive, and show different killing and sterilization susceptibility according to their development stage. It is generally accepted that chromosomal damage (structural and numerical anomalies) is the cause of dominant lethal mutations. Dominant lethal mutations occurring in a germ cell do not cause dysfunction of the gamete, but are lethal to the fertilized egg or developing embryo [16]. The earlier stages of spermatogenesis (spermatocytes and spermatogonia) are generally more radiosensitive than later stages (spermatids and spermatozoa). Dey and Manna [17] found that chromosomes in spermatogonial metaphase and anaphase I were more sensitive to X-rays than those in other stages. The larger the nuclear volume, apparently the greater is the sensitivity. Similar relationships were determined in animals and plants, and used to predict their sensitivity to chronic irradiation [4,18]. Furthermore, radiosensitivity appears to be influenced by additional parameters including cell repopulation capacity, tissue and organ regeneration ability, and biological repair (Harrison and Anderson 1996)"

Larval rearing and nutritional state

The nutritional state of pupae, or pre-irradiation starvation, may influence radiosensitivity [19–21]. It is therefore good to keep in mind that deviations in larval diet components and nutritional health may result in variations in dose-response to some degree, although these effects are not expected to be highly significant.

Pupa age

Generally speaking, older pupae tend to be more radioresistant than younger ones. In *Ae. aegypti*, there is a strong negative correlation (R^2 = - 0.95) between pupal age and

radiosensitivity [28]. It is therefore necessary to accommodate the age-related radioresistance with higher irradiation doses to achieve the target sterility. Pupae irradiated at 24 h of age and younger, also suffer greater somatic damage and present with significantly decreased adult longevity.

Sex

Regarding radiation induced sterilization, female arthropods are usually more radiosensitive than males [22,23], although there are exceptions. In *Aedes* spp, females are indeed more susceptible, as are female *An. arabiensis* [26].

[*excerpt from Bakri et al., 2005*] "Other insect models present a wide variation among species regarding relative radiosensitivity of males compared to females. This is probably due to factors such as differences in the maturity of oocytes that are present when the females are irradiated. For example, Mediterranean fruit fly female pupae that are irradiated two or more days prior to adult emergence, there is no egg production after irradiation at doses well below those needed to sterilize males. If, on the other hand, they are irradiated less than a day before emergence, females contain increasing numbers of oocytes that mature into viable eggs even if irradiated at doses sufficient to sterilize males".

Few studies exist assessing the full dose-response in female mosquitoes. We have seen that female *Ae. aegypti* pupae (>36 h old), cease to produce any eggs at a dose of 45 Gy (GC220, Co60, 80Gy/min IPCL, Seibersdorf, Austria) (Carvalho personal communication). *Aedes albopictus* females irradiated at pupal stage (aged 30-40 h) produced no eggs following a dose of 30 Gy (IBL 437, Cs137, Gy/min unknown, St Anna Hospital, Ferrara, Italy) [6], and female *An. arabiensis* pupae (20-26 h old) no longer produced eggs at a doe of 70 Gy (GC220, NICD, Johannesburg, South Africa), [26]. However, in this study, lower doses were not tested so it is unclear at what dose egg production is completely inhibited in this species. Depending on what information and for what purpose female dose-response is sought after, there is a need to investigate this aspect more closely to fully understand the biological effects.

Diapause (to be assessed)

There are differing reports on the effects of diapause on insect radio-sensitivity. In the codling moth *Cydia pomonella,* diapausing larvae were more radiosensitive than non-diapausing larvae [24] whereas other authors reported that radio-sensitivity in other species was not different in diapausing and non-diapausing larvae [25] and [27].

In mosquitoes, the effect of diapause on radiosensitivity has not yet been assessed. However, it is important to be aware of any diapausing behaviour in *Aedes* spp, as this can significantly affect egg hatch, and therefore hatch rates following irradiation experiments may be low, giving only the appearance that higher levels of sterility have been induced than is actually the case.

Geographic diversity

Genetic differences accounting for geographic diversity can contribute to differences in radiosensitivity in different strains but is not necessarily the case. Various strains of *Ae. aegypti* and *Ae. albopictus* and *An. arabiensis* that were collected at the ICPL, reared according to the standard guidelines, and irradiated using identical protocols did not show differences in radiosensitivity [28].

Physical factors

Ambient temperature

It has been suggested that lowering the ambient temperature during irradiation treatments reduces radiosensitivity, by reducing the insects' metabolic rate. This has yet to be assessed and confirmed for mosquito pupae, however maintaining consistency in all irradiation experiments in terms of temperature is good practice- not only for sterilizing pupae, but also for consistency and reliability in the dosimetry applied.

Ambient atmosphere

The oxygen levels, i.e. the atmospheric condition in which mosquito are subjected to before, and during radiation exposure can greatly influence the resulting induced sterility following irradiation, as is seen in other insects. Radiation effects are generally reduced in oxygen-poor environments (hypoxia) compared to oxygen-rich environments. Hypoxia describes an environment with 1-5% O_2, whereas normoxic conditions have 10-21% O_2.

[*excerpt from Bakri et al., 2005*] "Ionizing radiation initiates a chain of oxidative reactions, along the radiation path in the tissues, and the formation of free radicals, which in the absence of oxygen might be neutralized by combining with hydrogen radicals, resulting in no net damage. In the presence of oxygen, damaging peroxy-radicals may be formed, and the organic molecules, including the germ cell chromosomes, are irreversibly altered, e.g. dominant lethal mutations, leading to the production of imbalanced gametes. It must be noted

that high-LET radiation (e.g. alphas, neutrons) is less affected by the presence or absence of oxygen than low-LET radiation (X-rays and gamma radiation). This may be because high-LET radiation causes several ionizations within one macromolecule, damaging it beyond repair".

Dose-rate

To be added when data is complete and published

5. Post-irradiation assessments and Quality Control

The sterilization event is only one part of the sterile male production process, and this component of the SIT package for mosquitoes also requires and quality control (QC) to ensure that there are no unwarranted losses in the product (number of sterile males), and that the biological quality of the sterile adult male is high. The processes to obtain sterile males do not stop with irradiation. It is essential to ensure that the sterile males produced are also able to perform adequately once released, in order to maximize success rates in terms of mating performance in the field.

Various protocols and tools for the evaluation of adult male quality can be found in the "Guidelines for the quality control for sterile adult males" *(under development).*

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Appendix I: Protocol for the determination of dose-response for *Aedes aegypti* and *Ae. albopictus* following irradiation at pupal stage:

1. **Rearing:** Rear larvae following the guidelines for the Routine Colony Maintenance of *Aedes* spp. Try to synchronize hatching and rearing to get good proportion of pupation on the first day of pupation.
   Rear at low densities (~2 larvae/ml) to ensure good pupal size and male/female size difference
2. **Collect male pupae**: Collect pupae according to the below schedule to ensure pupal age lies within a 4 h (or maximum 6 h) window. Pupae should be more than 36 h old. If the strain being assessed has a pupal duration of less than 48 h, these collection times and irradiation times need to be adjusted so that no adults emerge during irradiation.
   a. **Calculate pupae age:** Clear the trays of the first pupae (for example at 8 a.m., or at 12 noon) and then allow for pupation. Then collect all pupae again after, for example, 4 h. These pupae will have a known age of 0-4 h. The next day at the time of first collection, they will be 20-24 h old. And on the 3rd day at the same time, 44-48 h old (see blue, or purples schedule 1 below).

Suggested schedule:

Day 1		Day 2		Day 3	
time	collect pupae	irradiate at:	pupal age	irradiate at:	pupal age
01:00		01:00		01:00	
02:00		02:00		02:00	
03:00		03:00		03:00	
04:00		04:00		04:00	
05:00		05:00		05:00	
06:00		06:00		06:00	
07:00		07:00		07:00	
08:00		**08:00**	20-24h	**08:00**	44-48h
09:00		09:00		09:00	
10:00	collect pupae	10:00		10:00	
11:00		11:00		11:00	
12:00		12:00		12:00	
12:00					
13:00		13:00		13:00	
14:00	collect pupae	14:00		14:00	
15:00		15:00		15:00	
16:00		**16:00**	20-24h	**16:00**	44-48h
17:00		17:00		17:00	
18:00		18:00		18:00	
19:00		19:00		19:00	
20:00		20:00		20:00	
21:00		21:00		21:00	
22:00		22:00		22:00	
23:00		23:00		23:00	
00:00		00:00		00:00	

3. **Collect female pupae**: on the 2nd day of pupation, collect pupae and sex male/female. Keep female pupae for mating. To ensure virginity of females, let female pupae emerge in single tubes, or small cups. Check emerged adults again to be sure they are female before mating them to males.
4. **Materials, doses and replications**:
   - Strain: *Aedes aegypti* or *Ae. albopictus* (record origin, and generation#)
   - Number of replicates ("reps"): at least 3 true reps (3 different cohorts, 3 separate irradiation events, 3 different times (dates), and 3 pseudo reps (3 groups of males from same cohort, to make 3 cages with mating and oviposition for each treatment and control).
   - Number of males: at least 30 per replication and per treatments and controls
   - Number of females: same as males (1:1 ratio)
   - Doses: for example, 0, 20, 40, 55, 70, 90 Gy
   - Dosimetry: (use available dosimetry)
   - Record info: irradiator type, source, loading date and dose-rate

Label all groups of males, cups, and cages!

True Repetition 1:

Cage ID	Pseudo-rep	**Dose (Gy)**	males/rep	virgin fem/rep
C1	1	**0**	30	30
C2	2	**0**	30	30
C3	3	**0**	30	30
1a	1	**20**	30	30
1b	2	**20**	30	30
1c	3	**20**	30	30
2a	1	**40**	30	30
2b	2	**40**	30	30
2c	3	**40**	30	30
3a	1	**55**	30	30
3b	2	**55**	30	30
3c	3	**55**	30	30
4a	1	**70**	30	30
4b	2	**70**	30	30
4c	3	**70**	30	30
5a	1	**90**	30	30
5b	2	**90**	30	30
5c	3	**90**	30	30
18 cages	**total pupae: >1080**		**540 males**	**540 females**

5. **Prepare your dosimetric films** for the exposures. See the guidelines for dosimetry on the IPCL website http://www-naweb.iaea.org/nafa/ipc/public/ipc-gafchromic-dosimetry-sterile-insect-technique.html. Use MD films for doses up to 90 Gy. Use HD film for doses over 90 Gy. (we have calibrated the films to accommodate these doses).
6. **Irradiation**: For each replication, place the groups of 30 male pupae in the center of a petri dish. (You can make a circle using silicon or glue to keep the pupae in the center). Pupae should be placed in a single layer and not overlap. Aspirate any excess water with a pipette, so that pupae stay damp, but are not submerged in water. Stack empty petri dishes with your sample in the middle, to ensure the placement of the sample in the middle of the gammacell chamber. Add your dosimeters near your sample. Remove the sample from the irradiator after exposure, and place pupae into labelled cups. Repeat with all reps, for all doses.

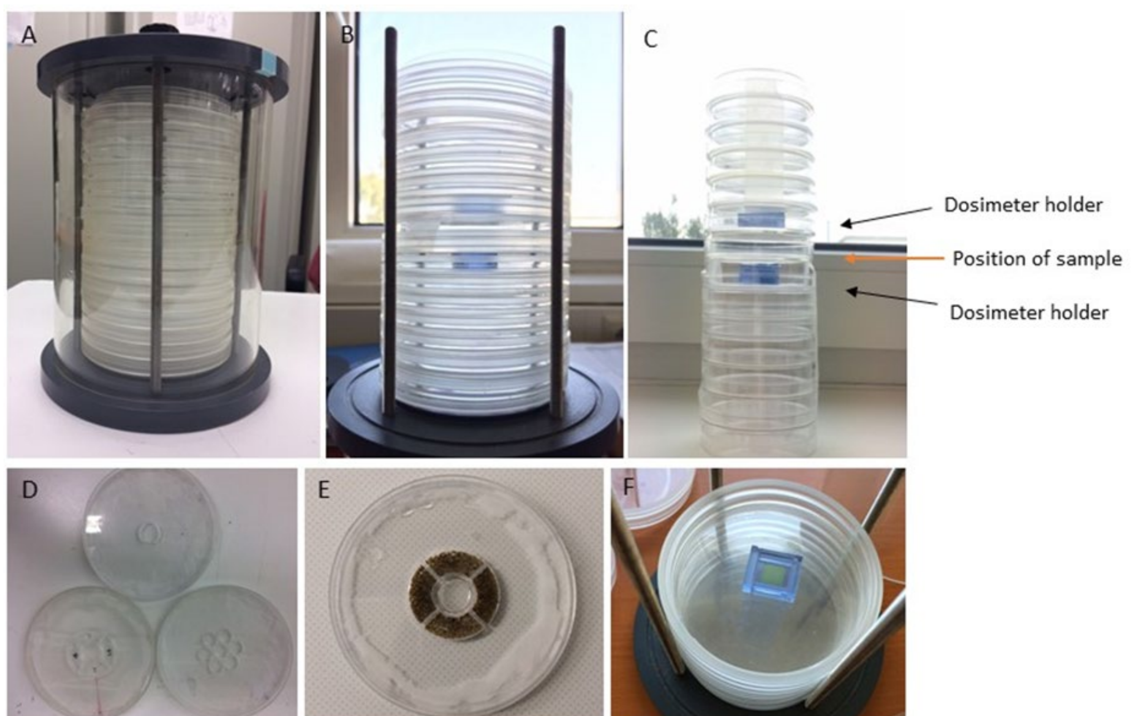

Figure 1. Examples of purpose-made petri dish holders for the standardized irradiation of mosquito pupae. Pupae are placed in the middle of the dish (Figures D & E), and dosimeters above and below the samples (figures B,D & F). Adequate build-up material is provided by a >4mm PMMA surrounding the sample (figure A).

Figure 2. Examples of samples placed in the center of the chamber (in small cups, left, of in petri dishes, right). These are surrounded by a 4mm thick PMMA tube, which provides adequate build-up material. The temperature is taken before and after exposure. The temperature information is important for the dosimetry

7. **Setting up your cages**: After irradiation, return the pupae to the insectary and let each group/rep emerge into separate cages with sugar feeders. Once adults have emerged, remove the cups and count and record any dead pupae. Record data in data sheet provided). Clean and keep the labelled cups to use them as oviposition cups later on for step 9. Remove any females.
8. **Mating:** Add virgin females (after being absolutely sure that they are virgin, and female) to the cages at a 1:1 ratio. Let them mate for 3 days (for example over the weekend- see suggested schedule).
9. **Blood feeding:** Blood feed the cages on 2 consecutive days (day 4 and 5 post-emergence).
10. **Oviposition:** add oviposition papers to the labelled cups (already provided from step 5), add water up to half of the paper, and add the cups to the cages. Collect the cups and egg papers (according to the schedule provided). Remove water from the cups but try to keep all of the eggs (example: pour water into a clean container and return any loose eggs to the egg cup with a pipette or brush. Do not contaminate with eggs from other treatment group).
11. **Egg maturation and storage:** leave the damp egg papers in the cups and place them in a tray/box with a lid for 2 days. Then open the lid slightly and allow for the papers to slowly dry. Place a net over the box to avoid free flying mosquitoes to lay eggs in your samples.
12. **Egg hatching:** Hatch egg papers after 14 days, using hatching methods described in the guidelines for the Routine Colony Maintenance of *Aedes* spp. Allow 2 days for the eggs to hatch.
13. **Record data**: Pour the contents of the cups into a small tray. Count all L1 larvae and record data into the datasheet provided. Count all hatched, and un-hatched eggs on the paper (and any loose eggs also) using a stereomicroscope. Record the data.
14. **Bleach eggs**: To check the un-hatched eggs for the presence of embryos, pour some bleach (example: Clorox, or any cleaning bleach (Sodium hypochlorite)) onto the egg paper using a pipette, and let the bleach dissolve the egg chorion. (this takes around 5-10 min-depending on the bleach concentration- try this on some regular eggs first for practice). Check the egg papers under the stereoscope and count any embryos present. They are small, almost clear in color, and hard to find/see. (see picture provided below).

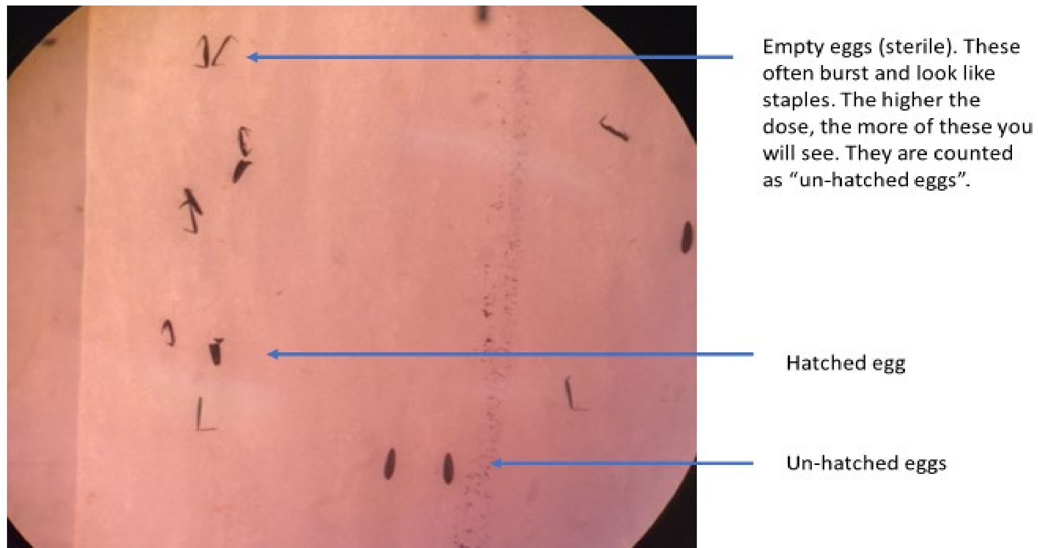

Figure 3. Hatched, un-hatched, and empty/burst eggs.

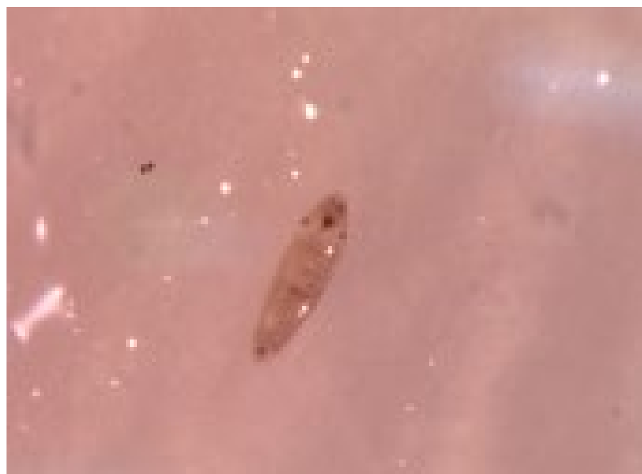

Figure 4. Determining the presence or absence of embryos. After bleaching the eggs. The egg chorion dissolves and leaves the embryos. These can be hard to see, but can be identified by their eye spots

Suggested schedule for the experiment:

SCHEDULE/Date	LAB
Strain:	*Aedes spp*
Tuesday	**Hatch**
Wednesday	rear
Thursday	rear
Friday	rear
Saturday	rear
Sunday	rear
Monday	**collect male pup (ex. 12-16:00) or**
Tuesday	**collect male pupae (ex. 10-14)**
Wednesday	(sex and tube females)

Thursday	**irradiate males**
Friday	add females
Saturday	mate
Sunday	mate
Monday	mate
Tuesday	Bloodfeed 1
Wednesday	Bloodfeed 2
Thursday	add egg cups
Friday	oviposition
Saturday	oviposition
Sunday	oviposition
Monday	collect egg cups
Tuesday	mature eggs
Wednesday	mature eggs
Thursday	slow-dry
Friday	slow-dry
Saturday	slow-dry
Sunday	Store eggs
Monday	Store eggs
Tuesday	Store eggs
Wednesday	Store eggs
Thursday	Store eggs
Friday	Store eggs
Saturday	Store eggs
Sunday	Store eggs
Monday	hatch
Tuesday	hatch
Wednesday	count L1, count hatched/unhatched eggs
Thursday	bleach eggs/count embryos

Appendix II: Protocol for the determination of dose-response for *Anopheles arabiensis* following irradiation at pupal stage:

1. **Rearing**: Rear *An. arabiensis* larvae until first pupation according to the Guidelines on *Anopheles* (mass) rearing. Try to synchronize hatching and rearing to get good proportion of pupation on the first day of pupation.
   Rear at low densities (~1 larvae/ml) to ensure good pupal size and synchronized pupation.
2. **Collect male/female pupae**: Clear trays of pupae at for example 12:00, and collect all pupae at 16:00 (for irradiation of 20-24h old pupae on following day at 12:00). Or see schedule and adjust pupa collection and irradiation time as required. Ideally pupae should be older than 20 h.

Day 1		Day 2		Day 3	
time	collect pupae	irradiate at:	pupal age	irradiate at:	pupal age
01:00		01:00		01:00	
02:00		02:00		02:00	
03:00		03:00		03:00	
04:00		04:00		04:00	
05:00		05:00		05:00	
06:00		06:00		06:00	
07:00		07:00		07:00	
08:00	collect pupae	**08:00**	20-24h	**08:00**	44-48h
09:00		09:00		09:00	
10:00		10:00		10:00	
11:00		11:00		11:00	
12:00		12:00		12:00	
12:00	collect pupae				
13:00		13:00		13:00	
14:00		14:00		14:00	
15:00		15:00		15:00	
16:00		**16:00**	20-24h	**16:00**	44-48h
17:00		17:00		17:00	
18:00		18:00		18:00	
19:00		19:00		19:00	
20:00		20:00		20:00	
21:00		21:00		21:00	
22:00		22:00		22:00	
23:00		23:00		23:00	
00:00		00:00		00:00	

3. **Sex pupae**: using a stereo microscope, sex pupae and separate males from females. Count batches of males for each sample/treatment group. Place female pupae into individual tubes for emergence. Once adults, check them again to be sure they are female before using them for mating them to males.
4. **Materials, doses and replicates**:
   - Strain: *Anopheles XXX* (record origin, and generation#)
   - Number of replicates (“reps”): at least 3 true reps (3 different cohorts, 3 separate irradiation events, 3 different times (dates), and 3 pseudo reps (3 groups of males

from same cohort, to make 3 cages with mating and oviposition for each treatment and control).

- Number of males: at least 20-30 per rep and per treatments and controls
- Number of females: same as males (1:1 ratio)
- Doses: 0, 20, 55, 70, 90, 110 Gy
- Dosimetry: (use available dosimetry) MD film and HD film
- Record info: irradiator type, source, loading date and dose-rate

Label all groups of males, cups, and cages!

True Repetition 1:

Cage ID	Pseudo-rep	**Dose (Gy)**	males/rep	virgin fem/rep
C1	1	**0**	30	30
C2	2	**0**	30	30
C3	3	**0**	30	30
1a	1	**20**	30	30
1b	2	**20**	30	30
1c	3	**20**	30	30
2a	1	**55**	30	30
2b	2	**55**	30	30
2c	3	**55**	30	30
3a	1	**70**	30	30
3b	2	**70**	30	30
3c	3	**70**	30	30
4a	1	**90**	30	30
4b	2	**90**	30	30
4c	3	**90**	30	30
5a	1	**110**	30	30
5b	2	**110**	30	30
5c	3	**110**	30	30
18 cages	**total pupae: >1080**		**540 males**	**540 females**

5. **Prepare your dosimetric films** for the exposures. See the guidelines for dosimetry on the IPCL website http://www-naweb.iaea.org/nafa/ipc/public/ipc-gafchromic-dosimetry-sterile-insect-technique.html. Use MD films for doses up to 90 Gy. Use HD film for doses over 90 Gy. (we have calibrated the films to accommodate these doses).
6. **Irradiation**: For each repetition, place the groups of 30 male pupae in the centre of a petri dish. You can make a circle using silicon or glue to keep the pupae in the centre. You can also put all 90 pupae for each dose in the same dish and split into groups of 30 before placing them into cages. Aspirate any excess water with a pipette, so that pupae stay damp, but are not submerged in water. Stack empty petri dishes with your sample in the middle, to ensure the placement of the sample in the middle of the gammacell chamber. Add your dosimeters near your sample. Remove the sample from the irradiator after exposure, and place pupae into labelled cups. Repeat with all reps, for all doses.

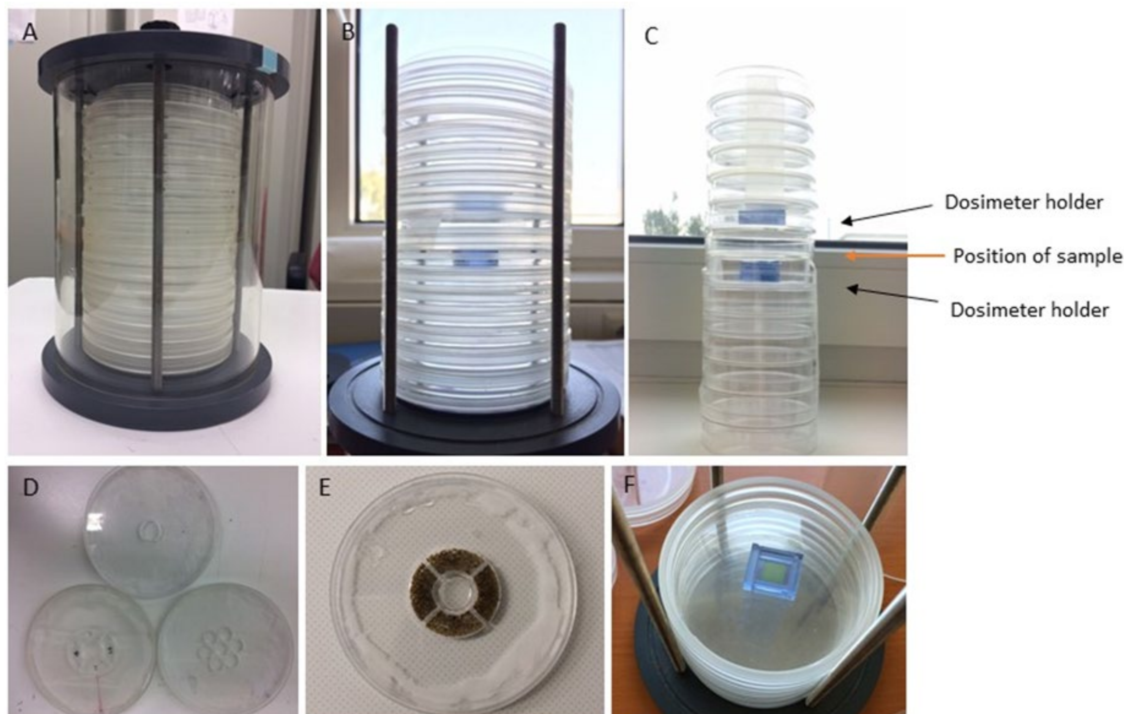

Figure 1. Examples of purpose-made petri dish holders for the standardized irradiation of mosquito pupae. Pupae are placed in the middle of the dish (Figures D & E), and dosimeters above and below the samples (figures B,D & F). Adequate build-up material is provided by a >4mm PMMA surrounding the sample (figure A).

7. Examples of purpose-made petri dish holders for the standardized irradiation of mosquito pupae. Pupae are placed in the middle of the dish (Figures D & E), and dosimeters above and below the samples (figures B, D & F). Adequate build-up material is provided by a >4mm PMMA surrounding the sample (figure A).
8. **Setting up your cages**: After irradiation, return the pupae to the insectary and let each group/rep emerge into separate cages with sugar feeders. Once adults have emerged, remove the cups and count and record any dead pupae (record data in data sheet provided). Clean and keep the labelled cups to use them as oviposition cups later on for step 9. Remove any females.
9. **Mating:** Add virgin females (after being absolutely sure that they are virgin, and female) to the cages at a 1:1 ratio. Let them mate for 3 days (for example over the weekend- see suggested schedule).
10. **Blood feeding:** Blood feed the cages on 2 consecutive days (day 4 and 5 post-emergence)
11. **Oviposition:** add sponges and round filter papers to the bottom of the labelled cups (already provided from step 8), add water to wet the sponge and filter paper, and add the cups to the cages. Collect the cups and egg papers according to the schedule provided. Remove the sponge and filter papers with the eggs. Line the cups with strips of filter paper (chromatography paper), and water to about half of the paper, and rinse the eggs from the round paper into the cups to hatch. Add 1/2ml of larval diet and allow eggs to hatch over 2 days. Gently remove the chromatography paper so that the eggs stick to the paper and determine the hatch rate under a stereo microscope. Puncture any unhatched eggs with a dissection needle to determine presence/absence of an embryo. Also count L1 larvae and record data for each treatment group and rep.

Hatched, un-hatched, and empty/burst eggs:

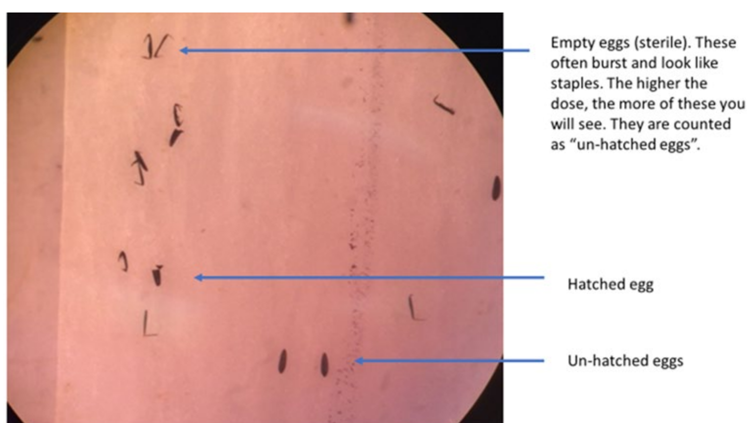

Figure 2. Hatched, un-hatched, and empty/burst eggs.

Suggested schedule for the experiment

SCHEDULE/Date	LAB
Strain:	*Anoheles spp*
Monday	**Hatch**
Tuesday	rear
Wednesday	rear
Thursday	rear
Friday	rear
Saturday	rear
Sunday	rear
Monday	rear
Tuesday	Rear (clear any early pupae)
Wednesday	**Ex. Collect pupae between 9:00-15:00**
Thursday	**Ex. irradiate males at 9:00**
Friday	Add virgin females
Saturday	mate
Sunday	mate
Monday	mate
Tuesday	mate
Wednesday	Blood feed 1
Thursday	Blood feed 2
Friday	Add egg cups
Saturday	
Sunday	
Monday	collect egg cups/hatch eggs
Tuesday	
Wednesday	Count L1, and hatch rates