Nuclear applications in insect pest control:

SIT for mosquitoes, vectors of human diseases

[Nukleare Applikationen in der Schädlingsbekämpfung: SIT gegen Stechmücken, menschliche Krankheitsvektoren]

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JLU Gießen
Faculty of Agricultural Sciences, Nutritional Sciences, and Environmental Management (FB09)
Institute for Insect Biotechnology
Department of Insect Biotechnology in Plant Protection (IBPP)
Winchesterstr. 2
35394 Gießen

Submitted by

Hanano Yamada
St.Louis, MO, USA

Gießen, 2023

Primary Supervisor: Prof. Dr. Marc F. Schetelig
Secondary Supervisor: Prof. Dr. Nikola-Michael Prpic-Schäper

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Prüfungskommission:

1. Gutachter(in):	Prof. Dr. Marc F. Schetelig
2. Gutachter(in):	Prof. Dr. Nikola-Michael Prpic-Schäper
Prüfer(in):	Prof. Dr. Miklós Bálint
Prüfer(in):	Prof. Dr. habil. Stefan Schippers
Vorsitzende(r):	Prof. Dr. Joachim Aurbacher

Tag der Disputation: [11/ 11 /2024]

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Hanano Yamada

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Contents

1.1. Summary

Mosquitoes are still the most significant killers on earth, as they transmit malaria and numerous arboviral diseases worldwide. Although significant efforts have been made to manage the spread and growth of mosquito vector populations, many conventional control tools have been proven ineffective, insufficient, or unsustainable, and alternative methods are needed to reach and maintain low vector densities to break the transmission cycle. The sterile insect technique (SIT) has been proposed as an additional tool to add to the area-wide integrated pest management (AW-IPM) strategy for the improved and sustainable management of mosquito vectors, as this technique has been shown highly effective against other insect pests by enabling total population control [1,2]

The essential components of the SIT include the mass rearing of the target species, the selection and reproductive sterilization of males, and their inundatory release into the target population, where successful copulation with wild females results in no offspring and consequently decreases the population size in subsequent generations. Key factors that influence the males' success in reducing the population size in the field include the level of sterility in the male mosquito and the biological quality and aptitude required for mating success once released [2].

Various methods have been used for achieving reproductive sterility in mosquitoes in the context of the sterile insect technique (SIT), such as the use of chemosterilants [3–9] or by modifying the insect's DNA [10–16]. However, the dominant approach for the SIT for most insect species is sterilization by exposure to ionizing radiation [1]. Most commonly, ^{60}Cobalt or ^{137}Caesium irradiators have been used for this purpose. More recently, the suitability of self-shielded X-ray irradiators as an alternative for sterilizing mosquitoes has been investigated [17,18]. However, a thorough evaluation should be performed on alternative devices before recommendations for the SIT can be made.

For the characterization of irradiators, including mapping the dose distribution in the irradiation chamber or the sample being irradiated, and for routine or experimental irradiation, dosimetry is an indispensable component of irradiation quality assurance. Therefore, a standardized, reliable dosimetry system is required for the work presented in this thesis and is the topic of the first chapter.

In response to reports from numerous SIT projects aimed to manage mosquito vectors that provided highly variable results regarding doses needed to achieve at least 99% induced sterility in the males, various endogenous and exogenous factors that could affect dose-response in the mosquitoes were assessed. The results indicated that the strain's geographic origin and pupal size did not affect dose-response. In contrast, pupal age, atmospheric conditions (hypoxia/anoxia), cold temperatures, irradiation dose rate, and sample preparation methods affected irradiation outcome. The realization following this series of experiments is that it will be logistically tedious to control for pupal age, and even more so to control the dissolved oxygen levels when irradiating large numbers of pupae in water. Thus, the focus of the studies shifted to irradiation at the adult stage to overcome these issues, develop

improved, standardized protocols for mosquito sterilization, and investigate irradiation protocols that may improve sterile male quality.

The final chapter of this work describes the application potential of an “off-the-shelf” blood X-ray irradiator as an alternative to self-shielded gamma irradiators for use in SIT programs. Careful dose rate measurements and dose mapping of the irradiation chamber showed an excellent dose uniformity and a sufficient processing capacity, capable of sterilizing 75 million adult *Aedes* mosquitoes week, proposing this technology a helpful tool and improvement for mosquitoes SIT programs in the future.

1.1. Zusammenfassung

Weltweit sind Moskitos noch immer für hohe Todeszahlen verantwortlich, denn sie sind in vielen Regionen Überträger von Malaria und verschiedenen Arboviren. Obwohl erhebliche Anstrengungen unternommen wurden, um die Ausbreitung und das Wachstum von Moskito-Vektorpopulationen zu kontrollieren, haben sich viele herkömmliche Kontrollwerkzeuge als ineffektiv, unzureichend oder nicht nachhaltig erwiesen, und alternative Methoden sind erforderlich, um niedrige Vektordichten zu erreichen und aufrechtzuerhalten und damit den Übertragungszyklus zu durchbrechen. Die sterile Insektentechnik (SIT) wurde als zusätzliches Werkzeug zur flächendeckenden integrierten Schädlingsbekämpfung (AW-IPM) vorgeschlagen, um eine verbesserte und nachhaltige Bekämpfung von Moskitovektoren zu ermöglichen, da diese Technik bei anderen Insektenschädlingen eine sehr hohe Effektivität bei der Kontrolle der Gesamtpopulation gezeigt hat [1,2].

Die wesentlichen Bestandteile der SIT sind die Massenzucht der Zielart, die Auswahl und reproduktive Sterilisation der Männchen und ihre flutartige Freisetzung in die Zielbevölkerung, wo eine erfolgreiche Kopulation mit wilden Weibchen zu keiner Nachkommenschaft führt und folglich die Populationsgröße in nachfolgenden Generationen verringert. Schlüsselfaktoren, die den Erfolg der Männchen bei der Reduzierung der Populationsgröße im Feld beeinflussen, sind das Maß an Sterilität bei den männlichen Mücken und die biologische Qualität und Eignung für den Paarungserfolg nach der Freisetzung [2].

Verschiedene Methoden wurden eingesetzt, um reproduktive Sterilität bei Mücken im Kontext der sterilen Insektentechnik (SIT) zu erreichen, wie z. B. der Einsatz von Chemosterilantien [3-9] oder die Modifizierung der DNA des Insekts [10-16]. Der häufigste Ansatz der Sterilisierung für die SIT bei den meisten Insektenarten ist jedoch die Bestrahlung mit ionisierender Strahlung [1]. Zumeist werden ^{60}Cobalt- oder ^{137}Caesium-Bestrahlungsgeräte verwendet. In jüngerer Zeit wurde die Eignung von selbstabschirmenden Röntgenbestrahlungsgeräten als Alternative zur Sterilisation von Mücken untersucht [17,18]. Vor Empfehlungen für die SIT sollte jedoch eine gründliche Bewertung alternativer Geräte durchgeführt werden.

Für die Charakterisierung von Bestrahlungsgeräten, einschließlich der Kartierung der Dosisverteilung in der Bestrahlungskammer oder der zu bestrahlenden Probe, sowie für routinemäßige oder experimentelle Bestrahlung, ist die Dosimetrie ein unverzichtbarer Bestandteil der Qualitätssicherung bei der Bestrahlung. Daher ist ein standardisiertes, zuverlässiges Dosimetriesystem für die in dieser Dissertation präsentierte Arbeit erforderlich und ist das Thema des ersten Kapitels.

Als Reaktion auf Berichte aus zahlreichen SIT-Projekten zur Bekämpfung von Moskitovektoren, die sehr unterschiedliche Ergebnisse hinsichtlich der benötigten Dosen für eine mindestens 99%ige induzierte Sterilität bei den Männchen lieferten, wurden verschiedene endogene und exogene Faktoren untersucht, die die Dosisantwort bei den Mücken beeinflussen könnten. Die Ergebnisse zeigten, dass Faktoren wie die geographische Herkunft des Stammes und die Puppengröße

die Dosisantwort nicht beeinflussten. Im Gegensatz dazu beeinflussten das Puppenalter, atmosphärische Bedingungen (Hypoxie/Anoxie), kalte Temperaturen, Bestrahlungsdosisrate und Probenpräparationsmethoden das Bestrahlungsergebnis. Die Erkenntnis aus dieser Serie von Experimenten ist, dass es logistisch aufwendig sein wird, das Puppenalter zu kontrollieren, und noch mehr, die gelösten Sauerstofflevel zu kontrollieren, wenn große Mengen von Puppen im Wasser bestrahlt werden. Daher verlagerte sich der Fokus der Studien auf die Bestrahlung im Erwachsenenstadium, um diese Probleme zu überwinden, verbesserte, standardisierte Protokolle für die Moskitosterilisation zu entwickeln und Bestrahlungsprotokolle zu untersuchen, die die Qualität steriler Männchen verbessern könnten.

Das letzte Kapitel dieser Arbeit beschreibt das Anwendungspotenzial eines „handelsüblichen" Blut-Röntgenbestrahlungsgeräts als Alternative zu selbstabschirmenden Gammabestrahlungsgeräten für den Einsatz in SIT-Programmen. Sorgfältige Messungen der Dosisrate und Kartierung der Bestrahlungskammer zeigten eine hervorragende Dosisuniformität und eine ausreichende Verarbeitungskapazität, die in der Lage ist, 75 Millionen erwachsene Aedes-Mücken pro Woche zu sterilisieren, und schlagen diese Technologie als hilfreiches Werkzeug und Verbesserung für Moskito-SIT-Programme in der Zukunft vor.

1.3 Justification of research

It has been the consensus that the same dose of radiation will produce the same, or close to the same sterility levels in insects within the same species, no matter what type of irradiator is used for the exposure. It has also been assumed that the same species will have the same or similar radio sensitivity no matter the geographic origin, handling protocols, and irradiation methods, resulting in straightforward protocols for insect sterilization. However, there is increasing incidence of diverging results obtained by different researchers regarding sterility in male mosquitoes induced by irradiation, despite using the same dose in the same species. Thus, there is a need to investigate these discrepancies and identify significant variable factors within the irradiation processes to develop optimal, standardized sterilization protocols in the frame of the sterile insect technique for managing mosquitoes.

2. Introduction

2.1 Mosquitoes: a major public health threat

Outbreaks and transmission of diseases by mosquitoes are often linked with climate. Tropical storms, prolonged rainy seasons and rising water levels provide abundant breeding sites. Shortened dry and cold seasons allow for better survival of the eggs, prolong mating seasons of the adults, and warmer conditions shorten the duration of the life cycle of the mosquitoes and the pathogens they carry, resulting in a population boom and enabling increased disease transmission as female mosquitoes digest blood meals faster and feed more frequently in warmer weather [19]. However, climate change is not the only driving force behind the surge in vector-borne diseases. Changes in land use and socioeconomic factors such as economic declines, population displacement, conflicts, the movement of pathogens by global trade and travel, the movement, and changes in habitation of wild animals, all appear to be equally crucial in the upward trend in the emergence and re-emergence of these diseases.

Mosquito-borne diseases are a major cause of human morbidity and mortality worldwide, severely affecting the economic development in already poverty-stricken communities. They are, however, not only a threat in tropical regions, but are moving into new territories due to climate change and globalization and are an imminent threat to immunologically naïve populations. Mosquitoes are responsible for more than 1 million human deaths per year and cause diseases and suffering in hundreds of millions more [20,21], and also transmit pathogens of veterinary importance.

Among the most treacherous of mosquito vectors, *Aedes aegypti* and *Ae. albopictus* are responsible for continuous transmission and outbreaks of several arboviruses, most notably dengue, chikungunya, Zika, and yellow fever. The worldwide risk and incidence of dengue has grown significantly in recent decades. Over 3 billion people (40% of the world's population) are at risk of contracting dengue. Currently, the WHO projects around 390 million dengue infections worldwide.

An estimated 500,000 people with severe dengue or dengue haemorrhagic fever require hospitalization each year, with a 2.5% fatality rate.

Chikungunya occurs in Africa, Asia, the Indian subcontinent, and to a lesser extent in the Americas. The disease has spread to Europe in recent decades, affecting several countries that have not seen chikungunya outbreaks before, such as Italy, France, and several Caribbean nations. The symptoms can be similar to dengue fever and are often misdiagnosed in regions where the two diseases coexist. There is no vaccine or cure for chikungunya, and treatment aims at symptom relief.

Rare sporadic cases of Zika virus disease have been reported between the 1960's to 1980's across the African continent and Asia, however more recently, larger outbreaks associated with the virus occurred in Micronesia (2007), then French Polynesia (2013) and most notably in Brazil in 2015 where it was linked to Guillain-Barré syndrome and congenital malformations in infants (Zika virus (who.int)). To date, mosquito-transmitted Zika infections have been reported in 86 countries.

Yellow fever is endemic in forty-four countries in Africa and Latin America, with a combined population of over 900 million. According to WHO estimates from the early 1990s, yellow fever will account for 200,000 cases and 30,000 deaths globally each year, of which 90% will occur in Africa [YellowFeverBurdenEstimation_Summary2013.pdf (who.int)].

Both *Ae. aegypti* and *Ae. albopictus* have been implicated in outbreaks of dengue, chikungunya and Zika. *Ae. aegypti* is confined to the tropics and sub-tropics, whereas *Ae. albopictus* can thrive in temperate and cold temperate regions. *Ae. albopictus* flourishes in a wide range of larval breeding sites, and the diversity of habitats explains the abundance of *Ae. albopictus* in rural as well as peri-urban areas. Although both species are highly anthropophilic, *Ae. aegypti* is more closely associated with human habits and often uses indoor breeding sites such as plant pots, flower vases, water storage vessels, latrines, and the same artificial outdoor habitats occupied by *Ae. albopictus*. This proximity of breeding sites to human habitats is a major risk factor for the diseases transmitted by these species and makes their populations challenging to suppress by conventional control tactics.

Malaria transmission is maintained by mosquitoes from the genus *Anopheles*, and is another major global killer, responsible for approximately 219 million malaria cases and an estimated 435,000 deaths annually (World Malaria Report, 2018). While malaria is generally a tropical and sub-tropical disease, spanning along the equator into central and northern South American continent, and India and Southeast Asia, the disease burden is heavily concentrated in sub-Saharan Africa, where about 90% of malaria deaths occur. Identifying potential habitats of the *Anopheles gambiae* and *funestus* complexes is especially important in African malaria and for predicting malaria incidences as these species are responsible for most transmission in that region. In some areas, more than one species is responsible for transmission, whereas in others, there is only one vector species, which provides an attractive target for genetic control efforts that focus on only one species at a time.

2.2 Control tactics

Ae. aegypti and *Ae. albopictus* immature stages develop in permanent and temporary artificial containers (water storage tanks, cisterns, water jars, animal drinking troughs, and discarded tires, water bottles, flowerpots) and small natural habitats such as tree holes, leaf axels, bamboo poles and many others. Larval control is based on removing such habitats (source reduction), using larvicides and releasing larvivorous fish and other predators. However, many of these larval sites are often cryptic or inaccessible, mosquito populations are becoming increasingly resistant to many larvicides, and community participation is difficult to attain. Space spraying with insecticides by thermal fogging or ultra-low volume (ULV) spraying is used to control adult *Aedes* vectors in response to arboviral disease outbreaks, to break the transmission cycle in the affected area (http://www.who.int/denguecontrol/arboviral/other_arboviral_chikungunya/en/). However, this method increases the selection pressure for insecticide resistance in the vector populations and can cause deleterious off-target effects on the environment and beneficial insects. Furthermore, this method is not effective against indoor-resting mosquitoes. Other protective measures against the vectors and disease transmission include mosquito trapping, insecticide-treated materials, and repellents.

The management of malaria vectors (*An. gambiae* and *funestus* complexes) is primarily based on larval source management, insecticide-treated nets (ITB), and indoor residual spraying (IRS) of insecticides. Although proven effective, this strategy has not completely inhibited malaria transmission, partly due to the development of insecticide resistance, but also because some species, such as *An. arabiensis* have shifted from indoor resting and biting, to resting and biting outdoors and thus escape the IRS [22,23]. Larviciding is not always applicable in certain larval sites such as small puddles, animal hoof prints filled with water, and water storage tanks [24].

2.3 SIT and AW-IVM Programmes

The abundant use of insecticides, including their monetary costs and costs in terms of human health and the environment, and the increased appearance of insecticide resistance in many insect pest populations, called for alternative management methods and led to the development of the “integrated pest management” (IPM) concept, which is defined as “the selection, integration and implementation of pest control methods based on predicted economic, ecological and sociological consequences” [25]. This means that the various control tactics are integrated into an IPM program in a way that the advantages of each method are exploited, and the disadvantages minimised to achieve the goals in an area-wide manner most efficiently. Simply mixing different control tactics does not constitute IPM. A complete understanding of the target insect’s biology and ecology and its interaction with other

organisms and the environment is required to optimise the timing, combination, interaction, and use of the various available control tactics.

Many effective AW-IPM programmes integrate a genetic control component. The term "genetic control" includes several methodologies, such as the sterile insect technique (SIT)[1], which uses irradiation (or chemosterilisation) to induce reproductive sterility. Other methods to induce sterility in a pest population include the release of insects of closely related sub species that result in hybrid sterility after mating with individuals of the target population, or insects with translocations or other chromosomal aberrations [2]. Transgenic approaches also provide additional options to the genetic control strategies [26], as do methods based on cytoplasmic incompatibility using *Wolbachia* infected mosquito strains [27–29].

The SIT is further defined as a method of pest control using area-wide inundatory releases of sterile insects to reduce fertility of a field population of the same species [30]. The first proof of principle of the SIT in a field setting was demonstrated by eradicating the New World screwworm (NWS) *Cochliomyia hominivorax* from the island of Curaçao in 1954 [75,82]. The subsequent eradication of the NWS from southern USA, Mexico, Central America and Libya remains the biggest success story in eradication campaigns to this day [31,32]. The SIT has celebrated other successes in controlling insects of agricultural and veterinary importance, such as fruit flies [33], moths [34], and tsetse flies [35].

For the implementation against mosquitoes, the technique consists of several basic components, including colonization and mass rearing of the target species; the separation of sexes and elimination of the potentially disease-transmitting females; the sexual sterilization of the males; handling, packing and transportation of the sterile males; release of sterile males over the treatment area; process and product quality control; and field monitoring following releases. Much progress has been made in the recent decade to develop and improve equipment and protocols for each component towards implementing the SIT against the mosquito vector species *Ae. aegypti*, *Ae. albopictus* and *An. arabiensis* at the FAO/IAEA Insect Pest Control Laboratory in Seibersdorf, Austria. The major achievements are reviewed by Vreysen et al., [36]. Many hurdles encountered for each of these components have been tackled, however, several challenges and bottlenecks have yet to be overcome.

Colonization and artificial rearing of mosquitoes is unique for each species and comes with individual challenges. For example, an artificial rearing environment can lead to selective pressures and changes in behavioural and physiological traits. For instance, following a few generations in the insectary, *An. arabiensis* males showed an acceleration of sexual maturation, able to mate with females only 11 hours after emergence, compared to 24 hours which were generally required in the wild [37]. Artificial environments can also hamper the swarming and mating behaviour of *An. arabiensis*, for example, if the cage netting is too dark (even beige), while *Ae. albopictus* and *Ae. aegypti* are picky about blood meals- refusing blood that has been frozen. Blood feeding rates can be affected by the size and shape of the cages, especially in mass-rearing settings, and the quality of the

blood and any chemical contaminants can quickly destroy the colony. A unique challenge of mass-rearing mosquitoes is that immature stages require water for development, often in countries where water is scarce or of unstable quality. Recently, the effect of water quality on mosquito mass-rearing parameters was investigated. The results showed that tap water with hardness/electrical conductivity beyond certain levels (140 mg/L CaCO3 or 368 µS/cm) was shown to harm the rearing production of *Ae. albopictus* and *Ae. aegypti* [38]. Large efforts have been made to find a suitable larval diet that will produce healthy adults, but will remain cost-effective [39–41]. However, some ingredients are hard to attain locally. Methods to quantify and aliquot eggs, synchronize pupation, collect pupae, stock cages with an optimal male:female ratios, maximise blood feeding rates, collect males and immobilize, pack, mark, and ship them, and finally release them in the field by ground or by drone have been developed [36]. Some quality control tests and tools have also been developed, optimized and protocols published [42–44]. The most critical bottleneck for the mass production of (male) mosquitoes is the sex separation or elimination of females for a male-only sterilization and release. Currently, there is no effective genetic sexing strain (GSS) by which females can be reliably eliminated from the release material. Some have existed but have largely relied on insecticide resistance [45]. The ANO IPLC1 GSS for *An. arabiensis* was based on (male) resistance to dieldrin. However, it was discovered that the treatment of eggs or larvae with this highly persistent organochloride resulted in the retention of the chemical residues by the surviving males, and these residues could accumulate in natural predators and the environment [46]. Thus, the GSS was not recommended for large-scale use in the SIT. Some progress has been made in the development of a GSS for *Ae. aegypti*, where a strain is now available with a red-eye mutation in females [47]. However, an efficient, automated method to recognize and separate the red and black eye individuals has not yet been developed. The possibility of using a transgenic *An. arabiensis* strain in which males carry a fluorescence gene was investigated, and life history traits and mass-rearing potential evaluated [48]. The sex separation in this strain can be performed by a large-particle flow cytometer (Complex Object Parametric Analyzer and Sorter (COPAS)) at the L1 stage, with high processing efficiency and accuracy. However, many countries are still unclear in the regulations (or lack thereof) on the release of sterile, yet transgenic males. GSSs, where a conditional lethal mutation allows for the early elimination of females from the rearing, is still an unattained goal for the species described in this work.

The phased conditional approach (PCA) to integrate the SIT into existing mosquito control programmes was proposed, where support to subsequent phases is conditional to the completion of required activities in the previous phase. The phases described are Phase 0, the pre-intervention; Phase 1, baseline data collection; Phase 2, small-scale field trial; Phase 3, pre-operational and Phase 4, operational [49]. When the PCA was formulated, there were 13 trials worldwide where sterile mosquitoes were released, and 10 more projects, where baseline data was collected. *Ae. albopictus* populations were successfully suppressed in China [50], Italy [51], Mauritius [52], and Germany [53],

and *Ae. aegypti* in Brazil [54], Cuba [55] and Florida, USA [56]. First releases in South Africa and Sudan have also shown promising results for the effectiveness of the SIT against *An. arabiensis* (publications pending).

2.4 Irradiation, sterility & irradiators

The basis of the SIT is inducing sterility in the females of the wild target insect population. For this, competitive, sterile males are needed for release. In living organisms, cells that are mitotically active (such as stem cells and germ cells) are the most sensitive to irradiation as radiation exposure causes germ cell chromosome fragmentation, inducing dominant lethal mutations, translocations, and other chromosomal aberrations, which in turn lead to the production of imbalanced gametes and subsequently the inhibition of mitosis and death of fertilized eggs or embryos [57]. Differentiated cells are generally less radio-sensitive than germ cells, and therefore insects require much higher irradiation doses to cause lethality, than is needed to achieve sexual sterility. However, irradiation-induced somatic damage can be observed in downstream quality indicators such as longevity reduction, flight ability, and/or mating propensity.

Attaining sterility in male mosquitoes, for example, has historically been achieved by subjecting the males to chemosterilants with varying success [3] and the use of chemicals is no longer the preferred choice due to health and environmental concerns, especially when used in large SIT programmes [9,58–61]. Both high-energy particle and photon beams may induce sterility in insects. Particle beams that have been tested include electrons, protons and neutrons. More commonly, photons are used, i.e. either gamma-rays or X-rays. Gamma-rays are high-energy photons produced by radioactive nuclei, whereas the interaction of fast-moving charged particles with heavy atomic nuclei produces X-rays. Whenever a moving charge is accelerated (slowed down, speeded up, deflected), it radiates energy as photons.

The most commonly used method for reproductive sterilization in insects (including mosquitoes) is using isotopic sources for gamma irradiation (usually ^{60}cobalt or less often ^{137}caesium). There are several advantages of isotopic irradiators: Low running costs – once the irradiator is set up, it requires little input to keep it running. The main expense is the initial acquisition and replacement of the cobalt after about 10 years. Gamma-rays have high penetration so that large volumes can be irradiated at a time, and the product/sample can be irradiated inside packaging, simplifying handling, and reducing the risk of contamination. The irradiation treatment is easily controlled, i.e. once the dose distribution and dose rate characteristics of the irradiator are known, the only parameter that has to be controlled is the duration of irradiation. The high penetration, with, for example, double-sided (or all sides) irradiation, gives good dose uniformity. Unlike electron irradiation, there is no risk of an unirradiated zone in the middle of the sample if the sample thickness or density is higher than

expected. They are highly reliable, as the mechanics involved are straightforward, and it does not rely on complicated electronics. Isotopic irradiators have disadvantages: the capital costs are generally higher than X-ray irradiators. The source decays with its characteristic half-life and must be replaced periodically. This is a relatively complex procedure and can result in the irradiator being out of service for some time. The cost of the source replacement is high, currently around $5000/kCi. Radioactive sources can harm the operating and maintenance staff and the surrounding environment if not handled appropriately. Regulation of irradiators is very tight, and accidents are rare, but regulation is likely to get stricter still. The isotopes need to be transported for irradiators' initial loading and reloading. Transport regulations are very strict, making it difficult and expensive, and certain countries or regions will not allow the passing of isotopes through their territory (e.g. the Mediterranean basin). All isotopic sources are now viewed as potential security risks. If a source is misused, it could lead to extensive contamination. As a result, there are moves to reduce the number of sources, and to improve security for them.

Typically, two types of gamma irradiators are used in sterile insect release programmes: self-contained dry-storage and large-scale panoramic irradiators (Figure 1). Self-contained dry-storage irradiators are currently the most frequently used for insect sterilization. These irradiators house the radioactive source within a protective shield- usually lead. They usually have a mechanism to rotate or lower the sample canister from the loading to the irradiation position. The Gammacell220 (GC220) is an example of a self-contained dry-storage irradiator, and is used for most of the studies in this thesis.

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.

More recently, X-rays and high energy electrons (in this case “high” referring to 1-5MeV) are becoming viable and practical alternatives due to lower costs, and less stringent regulations regarding safety, procurement and transportation.

In the recent decade, X-ray irradiators have been procured more frequently for small to medium sized SIT programmes. Low-energy X-ray devices such as the Radsource 2400, Wolbaki X-ray, Raycell MK2, Gilardoni RADGIL2, and Cegelec blood irradiators have been implemented in mosquito SIT projects. These have lower penetration, moderate dose-rates and thus moderate processing time, however, some devices allow for several canisters to be irradiated simultaneously.

In irradiation studies, the primary parameter is dose. The absorbed dose achieved must be as close as possible to the target (expected) dose throughout the irradiated sample. Dose rates vary within the given irradiation volume of any irradiator, with the distance from the radiation source(s) and the attenuation of the radiation absorbed both within the sample and the sample container. Therefore, the dose rate must be checked throughout the sample for each load configuration (sample size, container shape, position and material) to determine the dose variation. Then the sample configuration can be adjusted to ensure the sample receives a dose within the desired range.

The dose rate varies considerably throughout the chamber in the Gammacell 220 used in this series of studies (and in most Gammacells). The top and bottom receives the lowest dose rate, while the middle periphery the highest- due to the positioning of the isotopic sources (Figure 2). Thus, for the irradiation studies described in this thesis, a reference position was determined (in the center of the chamber) where the dose variation was minimal and doses achieved were reliable and repeatable.

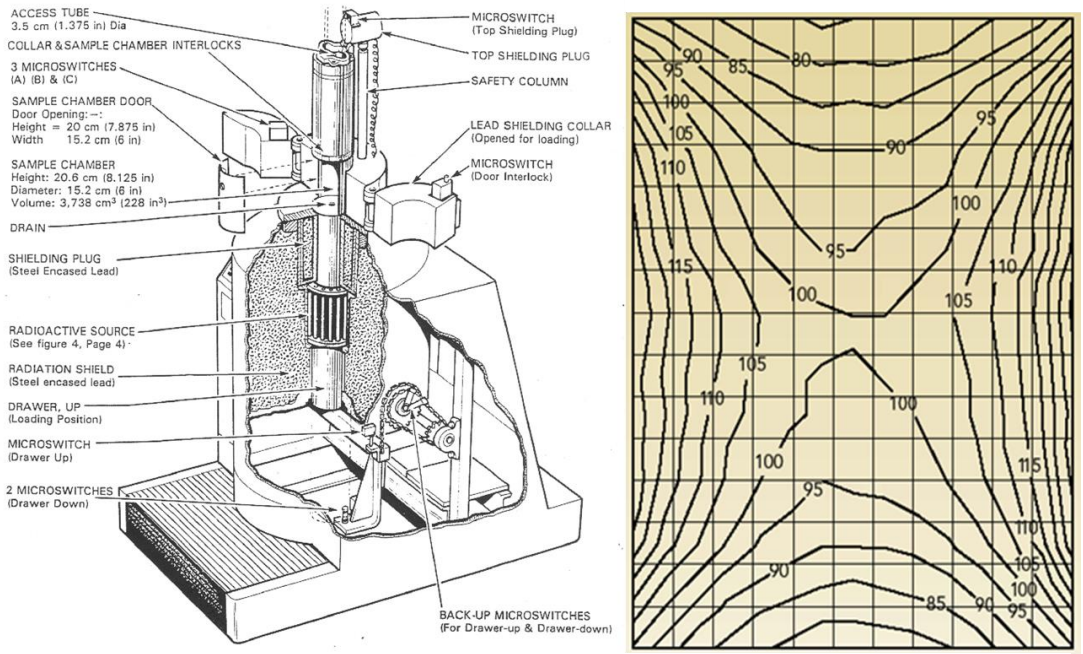

Figure 2. Diagram of commonly used Gammacell220. Left: Self-contained Gamma Cell 220 components and positioning of the isotopic pencils. Right: Dose distribution map of a typical GC220

Sample container shape and material must also stay consistent for experiments and routine insect sterilization. The shape and size of a canister can significantly improve dose uniformity (within

the canister and for the sample held within), as areas of higher or lower dose rates inside the irradiator chamber can be avoided. For gamma-ray irradiators, the container material and thickness of the canister walls will not affect the dose uniformity within the container, but it will affect the overall dose rate. However, in X-ray irradiators, the canister material does affect both dose uniformity and dose-rate. Different materials can change the photon spectrum by filtering out low-energy photons that do not reach the sample. A list of mass attenuation coefficients for various materials can be found on the NIST website.

Build-up material is also important for the standardized and uniform irradiation of samples. Build-up material surrounds the sample (for example mosquito pupae) and creates a standardized electron field. Two competing effects stabilize the electron field. These are the generation of high-energy electrons, which are dislodged from the material by incident photons, together with the decay of high-energy electrons as they interact with the material, and the high-energy electrons that can ionize further atoms in the material, thereby transferring part of their energy to a new electron, so creating a cascade of electrons with progressively lower energies to below the ionizing energy of the material and no further electrons are released. These many low-energy electrons (that can still ionize molecules in the insect tissue) cause the dominant lethal mutations, measured by the dosimeters. Thus, if the material of the canister is too thin, the electron equilibrium is not achieved and the electron cascade will continue through the sample, giving a rapidly changing dose rate in the first few millimetres of the sample. If the material is too thick, the equilibrium distance will attenuate the photon beam to an extent and reduce dose rate. For irradiating mosquito pupae in a gamma irradiator (^{60}Co), a 4 mm thick PMMA (e.g. Plexiglass, Acrylic, Perspex) layer surrounding the sample is an appropriate build-up material. This material is recommended as its behavior in response to radiation is similar to water (as are the pupae and other biological materials). In X-ray irradiators, less thick build-up material is required to reach an electron equilibrium (at 150 keV, 100 microns is already sufficient).

2.5 Importance of Dosimetry

Dosimetry is the process of measuring the dose applied to a sample. It depends on the radiation producing a measurable change in the dosimeter, proportional to the dose. The changes can be either chemical or physical. Dosimetry systems are used not only to verify the dose applied, but also to measure, for example, transit doses (in irradiators where sample chambers or the source itself is moved into position for irradiation), to map the dose distribution within the sample canister to ensure that all parts of the sample receive the necessary minimum dose and not more that the allowable maximum dose, to provide an estimate of the uncertainty in the dose measurements, to provide comparability between laboratories and between laboratory studies and field projects or

commercial applications, and to provide assurance to the relevant persons that the irradiation has been correctly performed.

For all irradiation studies and routine activities involving radiation sterilization of insects, dosimetry is a crucial component to ensure that the target dose is indeed administered, and the desired sterility reliably achieved, and is an integral necessity for the characterization of irradiation devices. Therefore, a reliable (and affordable) dosimetry system is required for routine quality assurance in irradiation processes. At the FAO/IAEA Insect Pest Control Laboratory, and many SIT programmes around the globe, a dosimetry system based on radiochromic (Gafchromic™ HD-V2 and MD-V3) films is used.

The Gafchromic films must first be calibrated using the type of irradiator used for the experiments (i.e. gamma-ray or X-ray). HD-V2 films are suitable for a dose range of 20 Gy to around 1000 Gy, whereas MD-V3 film is more suitable for doses below 100 Gy. In MD films, the active layer is sandwiched between two protective layers which makes is relatively resistant to water. However, the HD films have a protective layer only on one side and should not get damp. Both types of films can and should be protected by placing them into sealed plastic, aluminium envelopes, or paper envelopes (if not in contact with water), 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. Small PMMA dosimeter holders can be made or bought for exposures where this is not already available. The films should be read 24 hours after exposure, with an optical density reader.

2.6 Problem/Importance of standardization

Fundamentally, standardization means that SIT programme relevant personnel have an established, time-tested “process to use”. Well-developed and defined standardization can decrease ambiguity and guesswork, guarantee quality, and enhance productivity as work is done pre-defined and optimized. Standardization is critical to scientists and regulators to ensure the quality and interoperability of research processes, as well as the safety and efficacy of relevant personnel, and it is important for establishing the legitimacy of an area of scientific research, in this case, the irradiation-induced sterilization of male mosquitoes.

Consistent, reproducible, and reliable irradiation methods are required to ensure that millions of male mosquitoes reach the target sterility level over time, so that no unknown residual fertility levels can compromise the sterile males' beneficial effects. It is also essential to balance the high sterility levels targeted, with optimal irradiation and handling protocols to improve male biological quality by minimizing somatic damage and maintaining effectiveness in the field. The sum of the results reported in this thesis aims to define parameters important for the reliable, accurate, and reproducible induction of sterility in mosquito males, for the standardization of irradiation methods for effective SIT programmes.

3. Results

3.1 Gafchromic™ MD-V3 and HD-V2 film response does not depend on the temperature at time of exposure

First and foremost, a reliable dosimetry system is required for all activities involving irradiation to ensure that the desired dose is achieved. Gafchromic™ films are frequently used in SIT programmes and have proven easy to use and affordable for routine irradiation work. Following some inconsistencies in the dosimetry during experiments involving low temperatures, and newer film versions based on a slightly altered chemical composition, we investigated the films' response to temperature at exposure. Once the new versions of the Gafchromic films were assessed regarding response to temperature and new calibrations, we could complete the irradiation studies with consistent and reliable dosimetry.

Hanano Yamada* and Andrew G. Parker

Author contributions:

HY and AP both conceptualized, developed and performed the experiment protocol. HY and AP shared the writing and editing of the first draft, and later versions of the manuscript. AP performed the statistical analyses and provided the figures.

Status: Published in Radiation Physics and Chemistry, Annex 1

3.2 Identification of critical factors that significantly affect the dose-response in mosquitoes irradiated as pupae

The variation in irradiation-induced sterility in mosquito male pupae following radiation exposure documented in available publications and reported in different institutions and research groups has led to the main research questions posed in this dissertation. The aim is to identify factors that impact dose response in mosquitoes and to improve irradiation procedures, standardize protocols for replicability, and potentially improve sterile male quality by minimizing the off-target effects of radiation. The following study attempted to assess many of these factors and discover which are important for standardizing irradiation procedures.

Hanano Yamada1*, Hamidou Maiga, Jose Juarez, Danilo De Oliveira Carvalho, Wadaka Mamai, Adel Ali, Nanwintoum Severin Bimbile-Somda, Andrew Gordon Parker, Dongjing Zhang and Jeremy Bouyer

Author contributions:

HY conceptualized the series of studies and designed the experiments to assess effects of pupal age, pupal size and atmospheric conditions. HM and JJ conceptualized the design of experiments evaluating the effects of strain geographical origin and sample handling and preparation methods. HY, HM, JJ, DC and WM carried out the experiments. NSBS, WM and AA provided assistance to the insect rearing and experimental work and preparations. DC, HM, AP, DZ and JB contributed to the experimental designs and to the later versions of the manuscript. JB and HM carried out the statistical analyses. JB supervised and supported the project.

Status: Published in Parasites and Vectors, Annex 2

3.3 The role of oxygen depletion and subsequent radioprotective effects during irradiation of mosquito pupae in water

Following the previous study, it was observed that hypoxia had high radioprotective effects in all three species tested. However, there seemed to be differences between species in their tolerance to hypoxia, and the degree to which the dose response was affected. The following study attempted to better understand the oxy-regulatory behaviour in mosquito pupae when submerged in water and the subsequent dose effects in pupae subjected to hypoxia.

Hanano Yamada*, Hamidou Maiga, Nanwintoum Severin Bimbile-Somda, Danilo O. Carvalho, Wadaka Mamai, Carina Kraupa, Andrew G. Parker, Aiman Abrahim, Georg Weltin, Thomas Wallner, Marc F. Schetelig, Carlos Caceres and Jeremy Bouyer

Author contributions:

HY conceptualized the experimental designs for the experiments and drafted the original manuscript. CC assisted in developing the tools and methods for the irradiation in hypoxia. AP was responsible for the calibration, verification and assessments of the irradiator. DOC, MS and AP contributed significantly to the written content and later versions of the manuscript. GW and AA contributed to the design of the dissolved oxygen measurement experiment and equipment and provided materials including the calibration liquids and methods. CK, WM, NSBS and TW provided all live material following standardized rearing procedures and assisted in data collection. HY and HM carried out the experiments. JB and HM contributed to the experimental designs and carried out the statistical analyses. JB supervised and supported the project.

Status: Published in Parasites and Vectors. Annex 3

3.4 Radiation dose rate: a neglected critical parameter in dose-response of insects

Several critical factors that affect dose response in mosquito pupae have now been described and irradiation protocols improved to account for these factors. However, there seemed to be another factor that led to variations in irradiation outcome in terms of sterility achieved, even though the numerous variables had been controlled. Although dose has been, for the most part, considered to be a constant in insect sterilization, the next study investigates the effects of dose rate and the interaction of dose rate and dose on mosquito fertility.

Hanano Yamada*, Vanessa S. Dias, Andrew G. Parker3, Hamidou Maiga, Carina Kraupa, Marc J. B. Vreysen, Wadaka Mamai, Marc F. Schetelig, Nanwintoum S. Bimbilé Somda & Jeremy Bouyer

Author contributions:

HY conceptualized the experimental designs for the experiments and drafted the original manuscript. VD contributed significantly to the hypotheses on biological response mechanisms and written content of the original draft and later versions of the manuscript. AP was responsible for calibrating, verifying, and assessing the irradiator and irradiation processes and contributed to data analyses. CK and NS provided all live material following standardized rearing procedures and assisted in data collection. HY, HM and WM carried out the experiments. JB, MV, MFS and HM contributed to the experimental designs, contributed to data analysis, carried out the statistical analyses, and contributed to the formulation of hypotheses to explain all results, and contributed to the later versions of the manuscript. JB supervised and supported the project.

Status: Published in Scientific Reports, Annex 4

3.5 Effects of chilling and anoxia on the irradiation dose-response in adult *Aedes* mosquitoes

The studies on pupae irradiation have led to the conclusion that standardization of irradiation procedures will be difficult due to the short pupal duration of mosquitoes and pupa age effects, as well as the hypoxia effects when irradiating in water, thereby increasing evidence to support a shift to adult irradiation. Adult irradiation, especially when irradiating large numbers, requires their immobilization to avoid injury to the adults and to be able to compact them into limited space. The next study investigated the effects of chilling and anoxia on dose response and downstream effects on sterile male quality parameters.

Hanano Yamada*, Hamidou Maiga, Carina Kraupa, Wadaka. Mamai, Nanwintoum S. Bimbilé Somda, Aiman Abrahim, Thomas Wallner, and Jeremy Bouyer

Author contributions:

HY conceptualized the experimental designs for the experiments, carried out the experiments and drafted the original manuscript. HM carried out the flight tests and contributed significantly to the written content and later versions of the manuscript. AA contributed to the design of the anoxia setup and experiment and provided equipment and materials. CK, WM, NB, and TW provided all live material following standardized rearing procedures and assisted in the experiment and data collection. JB and HM contributed to the experimental designs and carried out the statistical analyses. JB supervised and supported the project.

Status: Published Frontiers in Bioengineering and Biotechnology, Annex 5

3.6 Radiation dose-fractionation in adult *Aedes aegypti* mosquitoes

Following the attempts to improve sterile male quality by irradiating adults in anoxia, and finding that longer exposures to nitrogen can negatively impact some quality parameters, other irradiation treatment protocols that have been shown to have positive effects on sterile male competitiveness were investigated. The most described strategy was to fractionate the irradiation dose into two or more smaller doses. We therefore evaluated the effects of 4 different dose fractionation treatments compared to the administration of one acute dose on downstream sterile male quality parameters.

Hanano Yamada*, Hamidou Maïga, Carina Kraupa, Nanwintoum Séverin Bimbilé Somda, Wadaka Mamai, Thomas Wallner, and Jeremy Bouyer

Author contributions:

HY conceptualized the experimental designs for the experiments, carried out the experiments and drafted the original manuscript. HM carried out the flight tests and contributed significantly to the data analysis and later versions of the manuscript. CK, WM, NSBS and TW provided all live material following standardized rearing procedures and assisted in the experiment set-up and data collection. JB and HM contributed to the experimental designs and carried out the statistical analyses. JB supervised and supported the project.

Status: Published in Parasite, Annex 6

3.7 Characterization and dose-mapping of an X-ray blood irradiator to assess application potential for the sterile insect technique (SIT)

Finally, we evaluated an X-ray irradiator, originally for blood irradiation, as a cheaper, off-the-shelf option for small to medium-sized SIT programmes for insect pests, as an alternative to gamma irradiators.

Yeudiel Gomez-Simuta, Andrew Parker 2, Carlos Caceres, Marc J.B. Vreysen, **Hanano Yamada***

Author contributions:

YGS, AP and HY conceptualized the experimental designs for the experiments, YGS carried out the dosimetry work, YGS, AP and HY drafted the original manuscript. CC and MV provided advice for the planning of the work and contributed greatly to the later versions of the manuscript. HY and MV supervised and supported the project.

Status: Published in Applied Radiation and Isotopes, Annex 7

3.8 Sterilizing insects with X rays or gamma rays - which irradiator to select?

The suitability of X- vs gamma irradiators for insect irradiation is an ongoing topic for discussion and seems to be far from settled. We therefore developed a comprehensive overview of the characteristics, advantages and disadvantages, and which irradiator to select for what kind of SIT programme in form of an opinion paper to assist during the decision-making process in SIT programmes when acquiring irradiation capacity to produce sterile male insects.

Hanano Yamada*§, Dongjing Zhang§, Andrew G. Parker, Marc JB Vreysen

Author contributions:

HY and DZ planned the development of the opinion paper. All authors contributed to the writing of the manuscript and provided their expertise on this subject.

Status: Published in Frontiers in Tropical Diseases, Annex 8

4. Discussion

Summary of results and implications

The controlled irradiation of the mosquito samples using accurate dosimetry was essential for verifying the doses used throughout the experiments described in this thesis and estimating the dose measurements' uncertainty. Numerous publications on Gafchromic films before 2010 have described the effects of temperature at irradiation on the response of the films. Thus, a temperature correction coefficient was incorporated into the dose calculation based on the films' optical density readings. Following several experiments involving cold temperatures at irradiation, the dosimetry results gave rise to doubts regarding the performance of the dosimetry system. The effect of temperature on the response of the newer versions of the Gafchromic films were thus investigated. It was found that temperature (within a range of 5-40°C) has little to no effect on the development of the films (i.e. the magnitude of increase (HD films) or decrease (MD films) in the film's response was less than 2% over the entire temperature range, compared to 20% over a temperature range of 14 - 44°C in the older versions of the films [62]). The overall uncertainty in Gafchromic film calibrations is typically under 4%. This includes the uncertainties from the transfer standard dosimeter (e.g. alanine or ion chamber), the non-homogeneity of a film production lot, and the readout temperature in the densitometer. Thus, over a moderate temperature range, a correction for temperature has become unnecessary as this uncertainty of less than 0.3%, when combined with the other uncertainties, would be negligible. The total uncertainty may increase over a large temperature range by around 0.6%. Thus, when large temperature differences are expected during irradiation experiments, a corresponding calibration of the film is recommended. However, for mosquito irradiation, temperatures below 5°C and over 40°C are likely never to be used as these lay outside the viable range for most species.

A datasheet provided with the dosimetric (HD) films includes a graph showing the spectrum of the film over a range of doses, whereas the MD films' data sheet no longer includes its spectrum's plot. However, the data sheet explains that the chemical composition of the MD film is the same as the HD film. The plot shows that the wavelength of the major and minor peaks shifted to shorter wavelengths as the dose increases (by approximately 10 nm), with the major peak of the MD film being near 635 nm. Our observations also found the major peak to be at 635 nm, but the shift of the peak is now much smaller (2 nm) towards longer wavelengths. We also found that the response of the HD film is unexpectedly high, which reduces the measurements' accuracy at doses over 100 Gy because the OD of the film approaches the maximum saturation of most measuring instruments at the wavelengths close to the peak.

Overall, apart from the high response of the film in the batch used for the current studies, it was found that the newer versions of the HD and MD films are highly improved, simplifying the use of these films for dose measurement. With the improvements and adjustments made for the Gafchromic dosimetry system, the effects of chilling, for example, during irradiation on mosquito dose-response, could be investigated while dose-effects could be ruled out and the results of the film development could be trusted.

Following the assessment of temperature effects during radiation exposure on the Gafchromic films, the question arose whether other factors, such as changes in ambient atmosphere could affect film development. Irradiation of insects in hypoxia is common and modified atmosphere packaging may be used for phytosanitary irradiation experiments, in which case the dosimeters may be placed inside the altered atmosphere. A series of experiments has been recently initiated to assess the behavior of Gafchromic films in anoxia, and different oxygen concentrations. Various mixtures of nitrogen and air at different ratios were prepared in gas tight bags and effects of irradiation on film response were assessed. Preliminary data have shown that reducing the oxygen concentration surrounding the films increases the films response. The effects are counterintuitive and additional experiments with altered atmosphere exposure durations are needed to explain the results adequately. The effects of temperature while reading the films in the densitometer have also been investigated and results are pending publication. Understanding the characteristics of the dosimeters used in radiation research is essential to ensure that the doses used are accurately verified and results reliable.

The series of studies assessing biological and physical variable factors during radiation exposures provided ample insight into several critical factors that affect dose response in irradiated mosquito pupae, and how deviating handling procedures and irradiation protocols can significantly alter resulting sterility levels in the release material [63]. This is crucial as over-dosing and under-dosing can result in uncompetitive or sub-sterile males, which could compromise the SIT in the field. In general, *Anopheles* spp. are more radioresistant than *Aedes* spp., requiring roughly double the dose for complete sterility [63]. However, within the same species, various strains with different geographic origins irradiated with the identical protocol and irradiator showed the same level of sterility at a given dose. Although similar insect strains generally maintain a similar radiosensitivity, strains reared in different locations with differing background (environmental) radiation levels may become more, or less sensitive to radiation treatments [64].

In all strains tested, pupal age was one of the most critical factors significantly affecting dose response. In *Ae. aegypti*, for example, there was a strong negative correlation (R^2 = -0.95) between pupal age and radiosensitivity, resulting in differences in residual fertility post-irradiation of up to 7% between males irradiated as young pupae (<10 hrs old) versus old pupae (> 40 hrs) [63]. This is crucial as the commonly accepted maximum fertility level of males released in the field is <1%. This

means that the radiation dose administered to a batch of pupae should be high enough to achieve >99% sterility in the oldest individuals. Although larval mass-rearing guidelines exist that aim for uniform larval development and synchronized pupation, obtaining all male pupae within an 8 h window for irradiation of uniformly aged pupae has not been possible. This implies that younger pupae within the sample batch are overdosed, incurring excess somatic damage and thus poor-quality adult males. Assessments of longevity of pupae irradiated at various ages showed a significant survival reduction in pupae under 24 hours of age.

Sex also determines relative radiosensitivity. In most arthropods, females are more sensitive to irradiation than males (although there are numerous exceptions, such as the American cockroach, ixodid ticks, and some *Coleoptera*). This may be due to differences in oocyte maturity during irradiation exposure [65]. In both *Anopheles* and *Aedes spp*., females require far less radiation dose for full sterility than their male counterparts. Additionally, preliminary data of ongoing experiments have indicated that females irradiated at male-sterilizing doses may show altered blood-feeding behaviour in *Aedes* spp., whereas this has not been observed in *An. arabiensis* yet. Irradiation can also change the vectorial capacity in disease-transmitting mosquito females [66], an important factor to consider when perfect sexing systems are unavailable and some females are released together with the sterile males.

The irradiation of pupae in small numbers can be done in wet or dry conditions. Those irradiated in wet conditions were less sterile overall, but the variation in individual sterility levels was much greater than those pupae irradiated in dry conditions, after which the induced sterility levels in the males were more uniform [63]. This shows that handling protocols can affect irradiation outcomes, and that water may indirectly provide radioprotection. Therefore, the subsequent experiments aimed to investigate the radioprotective effects of water during pupae sterilization. It was discovered that mosquito pupae, while submerged in water, continue to respirate, and consequently deplete the surroundings of dissolved oxygen (DO), creating a hypoxic environment. Hypoxia is known to be radioprotective, as it diminishes the oxygen effects during irradiation. This explains the decreased sterility levels in males irradiated in hypoxic water compared to males irradiated in normoxia (air). The radioprotective effects of hypoxia have been shown in several other insect species relevant to the SIT, in phytosanitary treatments, and also in the field of medical radiotherapy in which oxygen effects and radioresistance in hypoxic cells are well described, especially in tumor cells [reviewed in 46]. In the Mediterranean fruit fly (*Ceratitis capitata*), pupae are packed in airtight bags and allowed to respire until the oxygen is depleted before irradiation treatment. Although higher doses are required to achieve the target sterility, reducing oxygen effects results in more competitive males [68]. Similar studies showing positive effects of irradiation in hypoxia include those on *Bactrocera olea* [69] and *Rodnius prolixus* [70]. Although some older reports exist in which the effects of hypoxia in mosquito irradiation are studied, it is still unclear whether this has beneficial effects in

downstream male quality parameters as is described for the medfly. Irradiation of *An. gambiae* (pupae) and *Culex quinquefasciatus* (pupae and adults) in nitrogen were reported to have no obvious positive impact [71,72], while another study showed that *Ae. aegypti* (pupae and adults) irradiated in nitrogen with doses up to 100 Gy were as competitive as non-irradiated controls, whereas those irradiated in air showed a significantly reduced competitiveness, even at lower doses [73]. Further studies are still needed to assess the effects of a hypoxic environment on mosquitoes' quality parameters. Increased mortality was observed in pupae submerged in hypoxic water for extended periods, especially in *An. arabiensis*, suggesting that the stress of the treatment itself could harm the resulting adults and that these could outweigh any positive outcomes of the radioprotective effects of hypoxia in pupae.

More controlled experiments to better understand the respiratory behaviour of pupae of *An. arabiensis, Ae. aegypti* and *Ae. albopictus* showed that the pupae could continue respiration, presumably via cuticular respiration [74], when submerged in water in a closed container. Interestingly, the three mosquito species exhibited different oxy-regulatory behaviour. *An. arabiensis* presented as oxy-regulators, meaning that their respiration rates remain constant regardless of surrounding oxygen level, whereas the two *Aedes* spp. were shown to be oxy-conformers, as they could down-regulate respiration rates as oxygen levels decrease. The environmental history of these mosquito species could be an important factor that shapes their response to hypoxia. The differences in tolerance to low oxygen environments may be an evolutionary response, now reflected by their preferred breeding sites. *Aedes aegypti* and *Ae. albopictus* select breeding sites which are small, shallow, and often contain organic debris and thus microbial depletion of dissolved oxygen. Contrarily, this adaptation was not necessary for anopheline species such as *An. arabiensis*, which often select larger, cleaner bodies of water for breeding. In any case, the importance of these findings regarding male sterilization is that such behavioural factors also can affect the irradiation outcome and must be considered when developing standard protocols.

Although many important variables for uniform and reproducible irradiation dose-response can be controlled for the formulation of standardized procedures, measuring and controlling the atmospheric conditions around individual pupae within a sample batch is difficult, if not impossible [74]. Large quantities of pupae need to be held in water to avoid damage by crushing, and pupae in limited layers still create pockets of hypoxia within the sample batch, providing significant radioprotection to a subset of the individuals. It has been proposed to dry the pupae for irradiation. However, the effects of the desiccation process on downstream male quality parameters have yet to be assessed. This issue, confounded by the logistic difficulty to obtain all male pupae within an 8-hour window, and preferably during working hours, led to a shift towards the development of irradiation protocols in the adult stage, where obtaining males of the same age, and being allowed a larger window (e.g., 1-2 days old) is simplified, and radiation effects are much more uniform. However,

irradiating large numbers of adults, which also comes with challenges and requires immobilization and a good packing protocol to avoid mechanical damage to the fragile insects.

First, a dose-response comparison of adults and pupae was needed to confirm that no higher doses were required for full sterility in adults. It was found that for *Aedes* spp., adults had a similar radiosensitivity to late-stage pupae. Adults were slightly more sensitive than pupae aged 40 hr or older, but the differences were usually not statistically significant. As with pupae, large quantities of adults need compaction to enable efficient processing during irradiation. Thus, immobilization of the adults is essential to compact, transport, and irradiate in the self-contained irradiators. Chilling the adults prior to packing for subsequent irradiation is a viable and practical option for immobilization in mosquitoes, moths, and other insects [75], although the low-temperature treatments themselves sometimes led to adverse effects on flight ability and/or mating competitiveness [76–79]. Immobilization of insects (mosquitoes) can also be done with anaesthetics such as chloroform, carbon dioxide, argon, desflurane or other chemicals. Anoxia, for instance, by replacing air with nitrogen, has the added benefit of reducing oxygen effects and thus off-target radiation damage, as described above [72,73,80]. However, any negative effects of any anaesthetics still require careful evaluation. In our study, chilling at 7°C adequately immobilized adults, did not lead to significant differences in induced sterility, and did not negatively affect flight ability if a recovery phase was provided. The longevity of treated adults was also not compromised. Chilling for 2 hours prior to irradiation did, however, slightly reduce the sterility in adult (and pupae) males by around 3%, suggesting that perhaps a decrease in metabolic rate in the insects has a small radioprotective effect. Although 3% may seem low, if sterile male release programmes prescribe a sterility level of 99% or more, a 3% residual fertility in the release material may cause considerable controversy. In any case, in cold treatments, the effective temperature, duration of chilling, and the species and strains' natural cold tolerance will all affect the male mosquitoes' quality and need careful consideration when protocols are developed.

Nitrogen was also shown to be an effective anaesthetic and knocked down adult mosquitoes almost instantly when anoxia was reached. As with pupae, and as reported for several insect species, nitrogen (anoxia) had highly radioprotective properties and reduced sterility in adult *Ae. aegypti* males up to 14-fold compared to males irradiated with the same dose in air. Even when using a very high dose of 90Gy, males irradiated in nitrogen survived significantly longer than males irradiated in air-namely as long as untreated, fertile control males [75]. However, the treatment in nitrogen (in this case for 20 minutes) had negative impacts on flight ability (with or without irradiation), and males did not recover within the 2-day rest period as was seen in males that had been chilled. The few other publications that report the effects of nitrogen treatment in adult mosquitoes also did not find significant improvements in male quality indicators. However, more studies are needed to make definite conclusions regarding anoxia effects on male mating competitiveness and other quality parameters [72,73,81].

Once released, a high level of biological quality in the sterile males is required for their mating success in the field. Balancing irradiation efficiency (and production efficiency in general) with resulting male quality is one of the main challenges in the SIT. It is still unclear which stress factors are most important in reducing male quality, and what combinations of stress factors may further exacerbate this. Irradiation procedures (and irradiation exposure itself) are one of these main stress factors in the production of sterile males [75,82]. Improving irradiation protocols, such as fractionating the total sterilizing dose to 2 or more smaller doses, has been shown to, in some cases, greatly improve sterile male quality. Dose fractionation, for instance, significantly improved longevity in boll weevils [83]. Improved competitiveness was reported in the spotted bollworm after fractionated doses, whereas longevity and insemination capacity did not change. In the Indian meal moth, splitting the irradiation dose into 3 fractions improved longevity and mating propensity [84]. Fractionating a fully sterilizing dose in the West Indian sweet potato weevil maintained competitiveness for 12 days instead of just 6 days when given an acute dose [85]. Ducoff (1971) reported that the more the irradiation dose is fractionated, the better the survival in the confused flower beetle, and fractionating the dose in the presence of nitrogen greatly improved tsetse fly longevity [87]. In our study in *Ae. aegypti*, four different fractionation treatments (35 + 35 Gy, 10 + 60 Gy, and with either 1 or 2-day intervals) were compared to a single acute, fully sterilizing dose (70 Gy) in terms of longevity, flight ability and mating competitiveness. A fractionated dose was generally better than an acute dose regarding longevity and competitiveness, especially in groups that received two equal doses of 35 Gy, with a two-day interval, as opposed to one acute dose of 70 Gy. A two-day interval provided better recovery than a one-day interval in the longevity and flight ability tests. Another interesting result was that those males who received a fractionated dose (regardless of treatment type) induced 100% sterility in the mated females, whereas males receiving a single acute dose, although at low levels, still produced minimal offspring. One hypothesis is that sterility levels in some insects depend on the timing of radiation exposures, regarding the timing of spermatogenesis. If spermatids are fully formed, the effects of irradiation in either one acute dose, or several fractionated doses may not affect the final sterility level. Alexander and Bergendahl [88] proposed that oxygen is somehow released in the cellular components between the radiation doses, and thus increases radiation damage during the second dose. A test in which dose fractionation was performed in argon supported this hypothesis as it was found that there was less biological damage than when oxygen was present.

Another possibility is that the chromosome breakage and/or DNA repair mechanisms are affected, which also depends on the stage of spermatogenesis. In sperm reaching maturity, a higher (subsequent) dose may be needed to reach the target sterility [87]. In any case, the full sterility following fractionation is an added benefit for the SIT against mosquitoes.

Although historical publications on other insect species report different degrees of positive effects on various male quality parameters, none have been found that report negative effects. For mosquitoes, only a few fractionation treatments have been tested to date and many more dose, fractions, and interval combinations could be evaluated to develop protocols for improved male quality. However, the time and handling involved in adult mosquito irradiation procedures, coupled with the limited number of days the adult males can be stored before release in the field, suggests that dose fractionation involving more than 2 split doses and 2-day intervals are not recommendable in terms of process efficiency. Our study in Ae shows the marginal improvements in male quality parameters. *aegypti* in a laboratory setting may not outweigh the required additional labour, unless a significant increase in male mating competitiveness is proven.

The radiation dose rate is another critical yet overlooked factor affecting dose-response in mosquitoes (and other organisms). Following several experiments with seemingly contradictory results, it was discovered that dose and dose rate have a complex interaction. This interaction is not linear and dose rate effects (DRE) change with dose. Generally, at lower doses (<30 Gy) the DRE (i.e. sterility) increased when dose rate increased. Contrarily, at higher doses (>40 Gy), the DRE decreased as dose rate increased. We propose that direct and indirect dose effects are affected by dose rate, i.e. inverse dose rate effects. Reactive oxygen species (ROS) are generated as by-products of the normal metabolism of oxygen and in response to, for instance, environmental stress. When organisms are irradiated, ROS can be dramatically generated within cells, usually through the radiolysis of water, which can cause oxidative stress due to insufficient antioxidative protection, and, thus, significant damage to DNA and other macromolecules. In our simplistic model [89], assuming that radiation dose remains the same, we propose that the higher the dose rate after a certain threshold (in *Ae. aegypti* in our study, this was found to be between 20 and 40 Gy), the more ROS–ROS recombination occurs. After this threshold, radiation damage is attenuated by ROS–ROS recombination but can increase if direct damage (by increasing radiation dose) is increased. Although it is not fully clear what mechanisms drive the dose rate effects in mosquito dose responses, some clues suggest the existence of a dose rate "region of minimal mutability" in mosquito irradiation, suggesting that there may be a possibility to pair dose rate and dose, to optimize the response in a way that high levels of sterility and minimal off-target damage can be achieved, thereby producing a better quality sterile male. However, the main point of the study is that dose rate has been a neglected parameter in the SIT field and process quality control measures must consider the natural decay of isotopic sources, or changes in radiation devices. As the interaction of dose and dose-rate is complex [89], and may change in combination with other biological factors in the insects, periodic biological dosimetry tests must be performed to ensure that the males produced for release have reached the target sterility levels. This

applies to SIT targeted against mosquito species and SIT programmes against all other insect pests with an irradiation component.

One of the first clues to dose rates' role in differential sterility results was an irradiation and dose-response study in male mosquitoes using an X-ray irradiator. The doses needed for full sterilization seemed lower when using X-ray devices than gamma-ray irradiators with higher dose rates. In the last decade, there has been increased interest in using X-ray irradiators as an alternative to self-contained gamma-ray irradiators, due to the simplified regulatory and safety requirements, and lower purchase costs. Recently improved coaxial orthovoltage tubes provide increased dose rates (compared to traditional medical X-ray devices) and good dose uniformity, and thus allow adequate processing efficiency for small to medium-sized SIT programmes. As more modern blood X-ray irradiators fit the minimum requirements for insect irradiation, they may provide a viable, cost-effective option for the SIT. The Raycell model MK2 is one such X-ray unit. A full evaluation of the device included assessing the central dose rate, dose distribution mapping in the irradiation canister, and estimations of the processing capacity for fruit flies and adult mosquitoes. Dose rates were measured at 15 reference points within the canister, ranging from 7.2 to 8.2 Gy/min (where instant rice was used as dummy material). An overall DUR of 1.28, or 1.19 after excluding the highest and lowest 1% of the readings, meets the IAEA recommendations of staying under a 1.3 limit. With these preconditions, it was estimated that 75 million adult mosquitoes/week can be produced, when using the 2L canister, with a processing time of 12 minutes per load, (i.e. 5 loads per hour), in a 5-day week with two 6-hr shifts per day [90].

The physical characterization of the MK2 X-ray irradiator provides an initial evaluation of the application potential of this “off-the-shelf” blood irradiator as an alternative to self-shielded gamma irradiators for use in small to medium-sized SIT programs targeting mosquitoes, and other insect pest species [90]. A positive assessment of dose rate, dose uniformity, and calculations of processing capacities for insect samples gives young SIT programmes a sufficient and cheaper option for achieving irradiation capacity, which to date, has been a limiting factor for many mosquito control projects lacking funding capacities for the more expensive gamma-ray irradiators and the infrastructure required to house isotopic devices.

Following the physical evaluation of the MK2 blood irradiator, the irradiator was assessed in terms of biological effectiveness in three key insect groups targeted by the SIT, namely fruit flies (*Ceratitis capitata* and *Anastrepha ludens*), tsetse flies (*Glossina palpalis gambiensis*) and mosquitoes (*Ae. aegypti and An. arabiensis*), in comparison to gamma irradiation (Appendix II). It was found that the X-ray irradiator was equally efficient in inducing the desired sterility levels in all insects tested. In all cases, the resulting sterility was slightly higher than when irradiated with the gamma irradiators. This however, can likely be attributed to dose-rate effects as was found in our previous dose-rate studies [89]. The processing efficiency of the MK2 according to the different insect species’ requirements was

also calculated and the advantages and limitations of X-ray devices over gamma irradiators are discussed.

The MK2, like other X-ray sources, has several advantages over gamma irradiators: much lower capital and transportation costs, and much simpler regulation and access control. As X-ray relies on electrical power, no radiation is emitted when the power is switched off. Servicing and repairs are more straightforward as there is no radiation issue to contend with, and regular carriers can supply replacement tubes, whereas the supply of replacement cobalt-60 sources is expensive and problematical. Due to stringent regulations and rising costs, there have been an increasing number of cases of denial or delay of shipments of radioactive material Field [91–93]. The lower energy of X-ray systems means that it is much easier to block the radiation and thus does not require special infrastructure to house them, especially as most are self-shielded. Finally, the skills needed for handling high level radioactive sources are scarce, whereas the skills for handling high-voltage systems required for X-ray are available in most countries.

The disadvantage is if the electrical supply is not reliable the system will not function. All X-ray systems also require a cooling system to prevent the tubes from over-heating, which can be difficult in remote locations. X-ray dose rates are often much lower than isotopic irradiators, and the lower energy, and in some cases beam configuration, restricts the volume that can be irradiated.

In conclusion, X-ray systems such as the MK2 evaluated in our study offer advantages for small SIT programmes with their lower costs and simpler regulation, but they cannot compete with panoramic isotopic irradiators for larger programmes. However, as the discussions surrounding the suitability of X- vs gamma irradiators seems to be far from settled, a comprehensive overview of the characteristics, pros and cons, and which to select for what kind of SIT programme was provided in form of an opinion paper to assist SIT programme leaders in the decision making process when acquiring irradiation capacity to produce sterile male insects [91].

Application in the field

The results gathered in this series of studies have provided new insight into the radiation biology of mosquitoes and have eliminated some of the unknowns surrounding the nuclear components of the SIT regarding male mosquito sterilization and have provided solutions to many of the problems encountered in the numerous SIT irradiation facilities around the world. The investigations into variable factors during the irradiation process that could affect dose response have enabled the formulation of standardized guidelines for provision to FAO/IAEA Member States with an SIT programme against mosquito vectors, the main output of the research described in this thesis. The Guidelines are available on the IAEA website (Guidelines for Irradiation of Mosquito Pupae in Sterile Insect Technique Programmes | IAEA). An updated version that includes irradiation at adult stage and additional protocols is provided in Appendix I and will soon replace the current version on the IAEA

website. The protocols provided in the Guidelines have been adopted by many technical cooperation projects in Member States and has become the basis of IAEA/TC training on irradiation and dosimetry for the SIT package for mosquitoes.

Future research plans

Compact X-ray units are a suitable and cheaper alternative to self-shielded gamma irradiators for small- to medium-scale SIT and research for developing the SIT for new target pests (like mosquitoes). However, there are no adequate substitutions for industrial panoramic gamma irradiators for field application in existing, large operational SIT programs. There is a need to evaluate currently available alternatives and consider the fast-evolving industrial irradiation technologies that will not be a step back from gamma-based SIT processes, but a step forward. This includes the evaluation of existing and novel X-ray and electron beam technology, including flat panel X-ray conveyor systems and compact e-beam units (for example, Palletron, IBA Rhodotron) Flat panel X-ray; point source versus linear source instruments, and high-power accelerators with E-/X conversion versus low energy e-beam systems.

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Annexes 1- 8, and Appendices I & II

Annex 1: Gafchromic™ MD-V3 and HD-V2 film response does not depend on the temperature at time of exposure;

Annex 2: Identification of critical factors that significantly affect the dose-response in mosquitoes irradiated as pupae;

Annex 3: The role of oxygen depletion and subsequent radioprotective effects during irradiation of mosquito pupae in water;

Annex 4: Radiation dose rate: a neglected critical parameter in dose-response of insects;

Annex 5: Effects of chilling and anoxia on the irradiation dose-response in adult Aedes mosquitoes;

Annex 6: Radiation dose-fractionation in adult Aedes aegypti mosquitoes;

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Annex 8: Sterilizing insects with X rays or gamma rays - which irradiator to select?

Appendix I: Guidelines for irradiation of mosquitoes in sterile insect technique programmes;

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Gafchromic™ MD-V3 and HD-V2 film response depends little on temperature at time of exposure

Hanano Yamada [*], Andrew Parker [1]

Insect Pest Control Laboratory, Joint FAO/IAEA Centre for Nuclear Techniques in Food and Agriculture, P.O. Box 100, 1400, Vienna, Austria

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ABSTRACT

Controlled irradiation of insects is required for the sterile insect technique (SIT) as the insects require an optimal dose to ensure full sterility but overdosing must be avoided for the successful implementation of the SIT. Reliable dosimetry is thus necessary for irradiation quality assurance. Gafchromic™ films are frequently used in SIT programmes both for dose mapping and for dose confirmation.

Numerous publications prior to 2010 on the characteristics of Gafchromic films all show that temperature at irradiation has an effect on the response of the films. New versions of these films became available in 2010, with a yellow marker dye to allow correction for variation in the active layer thickness and reduced energy dependence in the films' response. As no information on the temperature effect have been published since the production of the new films, we completed a series of exposures of the new HD-V2 and MD-V3 films at varying temperatures and doses to evaluate the films' response. The MD-V3 and HD-V2 films have significantly improved temperature response at the time of irradiation and consistency of the absorption spectrum at different doses. These improvements lead to simplifications in the process of using these films for dose measurement.

1. Introduction

The Sterile Insect Technique (SIT) is a method of insect pest control based on rearing large numbers of insects, reproductively sterilizing them with ionizing radiation and releasing them into the wild to mate with the wild insects to reduce or eliminate their populations (Dyck et al., 2021). Dose control is important to ensure that the insects are appropriately sterilized without overdosing, which can lead to a loss in biological quality, or under-dosing, resulting in the release of partially fertile insects. The Gafchromic™ films MD-V3 (range 1–100 Gy) and HD-V2 (range 10–1000 Gy) (Ashland Advanced Materials, Bridgewater NJ, USA) are frequently used in SIT programmes both for dose mapping and for dose measurement/confirmation. Standard operating procedures (FAO/IAEA, 2022a; 2022b) for the use of these films are available that guide the user through the handling, calibration and use of these films for SIT, and also guide the user in the influence quantities that can affect the film response to the ionizing radiation. The assessment of influence quantities and the calculation of an uncertainty budget are covered in detail in ISO/ASTM 51261 (2013) and Sharpe and Miller (2009). For handling the insects during irradiation it is sometimes advantageous to chill the insects to between 5 and 10 °C to immobilize them, so correction for the temperature of the dosimeters during irradiation may be important to ensure accurate dosimetry. The reading of the films is done at room temperature in the laboratory and varies little.

Gafchromic™ films first came on the market in the 1990s. A number of papers were published in the 1990s and 2000s describing the properties of these new films, including several that reported the effect of temperature at the time of irradiation (Li et al., 1995, 2000; McLaughlin et al., 1991, 1996; Mincher et al., 1996) and two papers reviewed the available information (Niroomand-Rad et al., 1998; Soares et al., 2009). Rink et al. (2008) also measured temperature effects (in EBT film) but their results are complicated by the optical density readings that were taken simultaneously with the exposure so that reading temperature was also changing. The various papers all show that temperature at irradiation has an effect on the response of the Gafchromic films, but that this may vary with both dose and reading wavelength. McLaughlin et al. (1996, Fig. 5) show that for MD-55 film there is a small but significant rise in response from 10 to at least 40 °C at all of their reading wavelengths but the NMD-55 had an almost flat response from 10 to 40 °C, only rising at 50 °C. This contrasts with the older results of McLaughlin

* Corresponding author. Insect Pest Control Laboratory, P.O. Box 100, 1400 Vienna, Austria.
E-mail addresses: h.yamada@iaea.org (H. Yamada), a.g.parker@protonmail.com (A. Parker).
[1] Permanent address: Roppersbergweg 15, 2381 Laab im Walde, Austria.

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et al. (1991, Fig. 7) for the Gafchromic Dosimetry Media and the subsequent results of Li et al. (2000, Fig. 6) for Gafchromic DM-1260 (HD810), which both show marked increases in response with temperature from 20 to 50 °C and 14 to 40 °C, respectively. Li et al. (2000), however, show that while the response continues to increase over 40 °C when the film was read at 580 nm, when read at 400 nm it starts to fall.

In the SIT such high temperatures are not used, the practical range for insect handling is 0–30 °C. Normally the films are read at 580–600 nm, depending on the densitometer available to the programme, so the lower almost linear section of the 580 nm plot from Li et al. (2000) was used to calculate a temperature correction coefficient to apply to the readings.

In 2010 Ashland introduced new versions of the MD-55 and HD810 films, MD-V3 and HD-V2 respectively, with a yellow marker dye and reduced energy dependence. Since the introduction of these new versions more than 30 papers and books on radiochromic film dosimetry have been published, but none contains information on the effect of temperature at the time of exposure.

The data sheet accompanying the HD-V2 film includes an absorption spectrum for the film in the range 400–700 nm to show the effect of different doses (Ashland, 2021a). Two peaks are apparent, a major peak at about 670 nm and a minor peak at about 610 nm. The wavelength of these peaks, however, varies depending on the dose, with the peak shifting to shorter wavelengths as the dose increases from zero to 500 Gy. The data sheet supplied with the former MD-55-2 film also included an absorption spectrum, but this is no longer available on their web site and the one with the current MD-V3 film does not include this spectrum (Ashland, 2021b). The HD-V2 film data sheet, however, notes that whilst the active ingredient used in the HD-V2 film is the same as that used in the MD-V3, EBT-XD and EBT3 films, the crystalline form is different resulting in the major peak occurring at about 670 nm rather than 635 nm for the other films (the EBT3 and EBT-XD data sheets also do not include an absorption spectrum). Our assumption, therefore, is that the MD-V3 film should show a similar change in the wavelength of the major peak as the HD-V2 film but shifted down by 35 nm. Callens et al. (2016) includes an analysis of the effect of dose on the absorption spectrum of EBT3 film, but it is not possible to see from their figure (Callens et al., 2016, Fig. 4) if the peak wavelength is changing and they do not provide this data elsewhere. Much of this work on the various Gafchromic films has been summarised in review publications (e.g. Darafsheh, 2021; Das, 2018; Niroomand-Rad et al., 2020).

To date, few SIT programmes have used low temperatures during irradiation, and those that have either performed the dose mapping at room temperature or set up a calibration at their irradiation temperature. There has been a recent increase in interest in mosquito SIT, particularly following the Zika outbreaks in South America, and mosquitoes can be irradiated both as chilled adults and as pupae in water at room temperature so it would be convenient to have a single calibration to maintain that can be used at both temperatures. During a recent collaboration with a research programme in France to provide Gafchromic dosimeters to confirm dose rate and dose mapping in an irradiator at 6 °C, it immediately became apparent that the temperature correction in use resulted in serious errors in the dosimetry. We therefore exposed MD-V3 and HD-V2 films at a range of temperatures and doses to investigate further.

2. Materials and methods

2.1. Irradiator

A Nordion Gammacell 220 irradiator was used with a central dose rate to water of 56.5, 56.0 and 55.6 Gy/min with no load in the exposure chamber at the time of the three repetitions, due to the decay of the ^{60}Co source. Dose rates for the GC220 were assessed and verified using a Farmer type 0.18-cm^3 free air ionization chamber (10×6-0.18, RadCal Corporation, Monrovia, CA. USA) in conjunction with a digitizer and electrometer (AccuDose Model 9660A) as a reference dosimetry system to measure the dose rate and accumulated dose at a designated reference position. The ion chamber system was calibrated by the John Perry Laboratory (St George's University Hospital Trust, London) with traceability to the National Physical Laboratory, with a calibration factor of 1.0 and uncertainty of 3.3% (k = 2) in the energy range 40–1250 keV.

2.2. Film, exposure and densitometry

Gafchromic™ MD-V3 radiochromic film (Lot# 01222001, expiration date July 2022) and HD-V2 (Lot# 02202001, expiration date August 2022) (Ashland Advanced Materials, Bridgewater NJ, USA) were used in this experiment.

The films were cut into 1 cm × 1 cm squares from a single sheet of film for each repetition and were placed in the bottom centre of aluminiumised Mylar pouches (Aluminum Laminate Detector Pouch FWT-81, Far West Technologies, 330 S. Kellogg Ave Ste D, Goleta, CA 93117), which were then sealed with a heat sealer. Three MD-V3 and 5 HD-V2 films were used per treatment and per repetition, placed in separate pouches. The HD-V2 films, being asymmetric, were stacked in the pouch such that the active layer of each film was in contact with the backing sheet of the next dosimeter. Both the HD-V2 and MD-V3 film batches were exposed at increasing temperatures of 5, 10, 20, 25, 30, and 40 °C; MD-V3 films with doses of 8.3, 29.1, and 74.8 Gy, and HD-V2 films with doses of 29.1, 124.7 and 415.7 Gy. A total of three repetitions were performed on different days and using different sheets of film from the same batch.

The pouches with the films were placed in a preparation bath (2L Nalgene polycarbonate container) containing water at the target temperature to stabilize the films to each treatment temperature. They were then transferred to a dosimeter holder (custom made, Fig. 1) in a second, identical 2L Nalgene polycarbonate container, containing water at the target temperature for irradiation. The holder was designed to

Fig. 1. Holder used to position the sealed pouch containing the dosimeters at the centre of the canister in water for irradiation.

standardize the position of the dosimeters for each treatment and each repetition at the centre of the radiation field where the dose rate gradient is at a minimum. The attenuation of the radiation due to the water was 0.831 at the centre of the canister. The water temperature was adjusted by adding either crushed ice or hot water to the water in the containers. The temperature was measured using a K-type thermocouple thermometer (HI 93532R, HANNA Instruments, Woonsocket, Rhode Island), which was compared to a mercury in glass thermometer (-5 °C – 50 °C range, graduated at 0.2 °C). The end of the thermocouple was held in place at the location of the films. The thermocouple was threaded through the GC220 via the top cap during irradiation (Fig. 2). The temperature was taken before, during and after each exposure, and the average (effective) temperature was noted.

The films were kept at room temperature and read after 24h ± 10 min in an optical density reader (DoseReader 4, RadGen, H-1118 Budapest, Sasadi út 36, Hungary) following the suppliers' instructions. The reader recorded the optical density (OD) at 4 wavelengths with central wavelengths of 458, 532, 590, and 625 nm and full width half intensity (spectral half width) values of 20, 40, 16, and 37 nm, respectively. Neutral density films (nominal values 0.5, 1.0 and 2.0 OD) and 10 untreated films were read for controls for both MD and HD films.

Full spectra were read using a Synergy H1 microplate reader using a Take3 plate (BioTek, Winooski, VT, USA) at 2 nm intervals from a representative film from each treatment between 400 and 700 nm. Wavelength peaks were estimated by interpolation.

2.3. Statistics

Analysis was performed using RStudio version 2021.09.2.382 (RStudio Team, 2022) with R version 4.1.2 (R Core Team, 2021).

MD-V3 and HD-V2 films were analysed separately. OD values were expressed relative to the OD at 25 °C at each wavelength and dose within each repetition and wavelength as a discrete factor. Full models of relative OD against temperature, dose and reading wavelength and their interactions with replicate as a random effect were constructed using lme in the package nlme version 3.1-155 (Pinheiro et al., 2022) and reduced stepwise, comparing models by ANOVA and AIC with aictab in the package AICcmodavg version 2.3-1 (Mazerolle, 2020) as long as no significant difference was detected. To improve the fits, dose was transformed as dose$^{0.6}$ for the analysis of MD-V3 and as dose$^{0.3}$ for the analysis of the HD-V2 film. Multiple regressions were constructed using lme for each reading wavelength separately. The line graphics were produced using ggplot2 version 3.3.5 (Wickham, 2016).

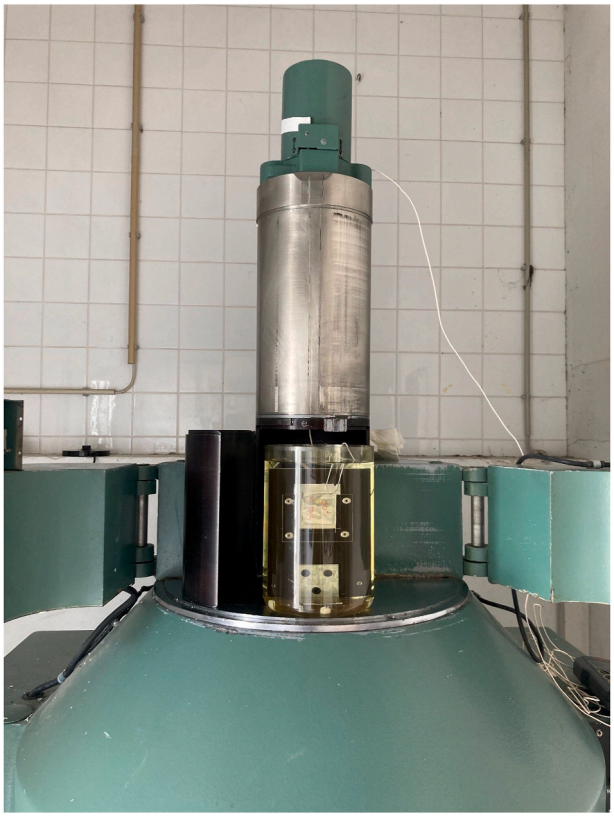

Fig. 2. Position of the thermocouple with its end held in place at the location of the films, and then threaded through the GC220 via the top cap during irradiation.

The original OD data set is available in **Supplementary File 1,** the relative OD data sets for the analysis of temperature and absorption spectrum OD in **Supplementary Files 2–6** and the R script file in **Supplementary File 7**.

3. Results

3.1. Effect of temperature on film response

The full model multiple linear regression models of relative OD against temperature (t_irrad), transformed dose (rdose) and reading wavelength (wvl) with the successive reductions are given in **Supplementary File 8** for MD-V3 and HD-V2. For the MD-V3 film the optimum model includes temperature, wavelength and dose with the interactions of temperature with wavelength and with dose. Removing wavelength as an explanatory variable significantly reduces the quality of the model (likelihood ratio 13.316, P = 0.038). In the optimum model (M3), temperature is not significant (t = 1.292, P = 0.197) but dose and the interaction of temperature and dose are both highly significant (t = 6.687, P < 0.0001 and t = -4.494, P < 0.0001, respectively). The wavelength 625 nm is near significant (t = -1.735, P = 0.0833) and the interaction of temperature with absorption at 590 nm is just significant (t = -1.993, P = 0.0467).

For HD-V2 film the optimum model (H3) also includes temperature, wavelength, dose and the interactions of temperature with wavelength and with dose. Removal of wavelength also significantly reduces the model quality (likelihood ratio 16.355, P = 0.012). Both temperature and dose are highly significant (t = -3.733, P = 0.0002 and t = -7.092, P < 0.0001, respectively). The wavelength 590 nm is significant (t = 3.032, P = 0.0025) and the interactions of temperature with 590 nm and with dose are significant (t = -3.433, P = 0.0006 and t = 8.156, P < 0.0001).

The complex interaction of relative OD with both reading wavelength and dose makes interpretation of the results difficult. We therefore analysed the data for each wavelength separately, adjusting for dose, to compare with the regression with all wavelengths.

For the MD-V3 film, when the interaction of temperature with dose is included in the analysis, the coefficient for temperature is positive for all measurement wavelengths but with only 425 nm being significant (Table 1). The magnitudes of the temperature coefficients are small, between 0.00003 and 0.00049. The interactions of temperature with dose are all negative for all reading wavelengths, with the interactions significant for reading wavelengths of 532, 590 and 625 nm. When the interaction is removed from the regression all the temperature coefficients are negative, between -0.00018 and -0.00073 and all significant except for at 625 nm.

The pattern for the HD-V2 film is very similar, but reversed with the coefficients for the interaction of temperature with dose at different reading wavelengths and the overall coefficient positive and the coefficients corrected for the interaction negative (Table 2). With the interaction terms, all the temperature coefficients are significant except for at 625 nm; without the interaction terms all are significant except for at 590 nm. Again, the coefficients are small, between -0.00017 and -0.00089 with the interaction terms, and between 0.00009 and 0.00047 without. The full analysis results are presented in **Supplementary Files**

Table 1
Coefficients for the regression of OD relative to 25 °C on temperature at time of exposure for Gafchromic™ MD-V3 film, adjusted for dose. Analysis was by the lme function of package nlme in R, including all interaction terms (upper section) or without interaction terms (lower section). Only the interaction temperature*rdose is shown here. Wavelength is the reading wavelength, All the full model with wavelength as a factor, the individual wavelength values from models without wavelength as a factor. rdose is the applied dose transformed as dose^0.6. For full results see **Supplementary File 9**.

Wavelength	Temperature			Interaction temperature*rdose		
	Value	t	P	Value	t	P
All	0.0000343	0.11518	0.9083 ns	-0.0000480	-1.46330	0.1439 ns
458 nm	0.0000358	2.20112	0.0292 *	-0.0000481	-1.39702	0.1644 ns
532 nm	0.0001179	0.41739	0.6770 ns	-0.0000720	-2.31098	0.0221 *
590 nm	0.0000246	0.08122	0.9354 ns	-0.0000924	-2.77314	0.0062 **
625 nm	0.0004880	1.65774	0.0994 ns	-0.0000819	-2.52278	0.0126 *

Wavelength	Value	t	P
All	-0.0004323	-6.46303	0.0000 ***
458 nm	-0.0003553	-2.56783	0.0112 *
532 nm	-0.0004676	-3.69457	0.0003 ***
590 nm	-0.0007275	-5.33123	0.0000 ***
625 nm	-0.0001782	-1.34625	0.1802 ns

Table 2
Coefficients for the regression of OD relative to 25 °C on temperature at time of exposure for Gafchromic™ HD-V2 film, adjusted for dose. Analysis was by the lme function of package nlme in R, including all interaction terms (upper section) or without interaction terms (lower section). Only the interaction temperature*rdose is shown here. Wavelength is the reading wavelength, All the full model with wavelength as a factor, the individual wavelength values from models without wavelength as a factor. rdose is the applied dose transformed as dose^0.3. For full results see **Supplementary File 10**.

Wavelength	Temperature			Interaction temperature*rdose		
	Value	t	P	Value	t	P
All	-0.0008877	-3.36641	0.0008 ***	0.0003109	5.40904	0.0000 ***
458 nm	-0.0008859	-2.88981	0.0042 **	0.0003107	4.64992	0.0000 ***
532 nm	-0.0007716	-3.19212	0.0016 **	0.0002710	5.14449	0.0000 ***
590 nm	-0.0009524	-3.44154	0.0007 ***	0.0002378	3.94220	0.0001 ***
625 nm	-0.0001679	-0.77026	0.4418 ns	0.0001205	2.53493	0.0118 *

Wavelength	Value	t	P
All	0.0003339	8.1501	0.0000 ***
458 nm	0.0004741	4.97480	0.0000 ***
532 nm	0.0004149	5.47292	0.0000 ***
590 nm	0.0000884	1.03909	0.2997 ns
625 nm	0.0003594	5.44664	0.0000 ***

9 and 10, respectively.

Figs. 3 and 4 present the OD over the range of temperatures 5–40 °C relative to the response at 25 °C at each dose and reading wavelength for MD-V3 and HD-V2 film, respectively, with the linear regression lines and 95% confidence intervals.

For the MD-V3 film the variation in response over the range 5–40 °C relative to the response at 25 °C is less than ±2% for all reading wavelengths for the middle and higher dose, with a tendency for the response to decrease with increasing temperature (Table 1). The lowest dose, 8.3 Gy, showed a different pattern with no effect of temperature except at 625 nm when the response rose slightly. For the HD-V2 film the variation in relative response at all temperatures and wavelengths was less than ±2%. At 124.7 Gy and 415.7Gy the response tended to increase with temperature, in contrast to the response of the MD-V3 films. At the lowest dose, 29.1 Gy, the relationship was again like the MD-V3 response with no significant change in response except at 590 nm when a slight decrease was observed.

The linear regressions of OD on temperature are significant, except at 625 nm for MD-V3 (Table 1) and 590 nm for HD-V2 (Table 2). However, the interaction of temperature and dose is significant at 532, 590 and 625 nm and for all wavelengths combined for MD-V3 (Table 1) and at 458, 532 and 625 nm and for all wavelengths combined for HD-V2, reflecting the difference in response at the lowest dose compared to the higher doses. The full data is available in **Supplementary File 1**.

3.2. Effect of dose and temperature on absorption spectra

The response of the HD-V2 film was unexpectedly high, with the gross OD exceeding the reading range of the spectrometer (OD = 3) for both the 124.7 and 415.7 Gy doses, despite the higher dose being less than half the stated maximum dose for this film. Analysis of the spectrum was, therefore, limited to the MD-V3 film.

Fig. 5 shows the wavelengths of the primary and secondary peaks at different doses for MD-V3 film. For the primary peak the wavelength increased by approximately 4 nm over the dose range used, and the secondary peak by less than 2 nm. Temperature at the time of exposure had no measurable effect on the wavelength of either peak (Fig. 6). The full data is available in **Supplementary File 2**.

4. Discussion and conclusions

The effect of temperature at the time of irradiation observed in this study was consistently small at the wavelengths measured. Figs. 3 and 4 clearly illustrate the complex nature of the relationship, with the regression of the relative OD on temperature not significant at the lowest dose and increasing with increasing dose, negative for MD-V3 and positive for HD-V2. In all cases the magnitude was small at less than ±2% over the complete temperature range 5–40 °C. This is in contrast to the reported temperature response of an earlier version of the Gafchromic HD-V2 film (DM-1260, later called HD810) (Li et al., 2000), which showed an almost 20% change at 580 nm over the range 14–44 °C. In operational SIT programmes the overall uncertainty of the

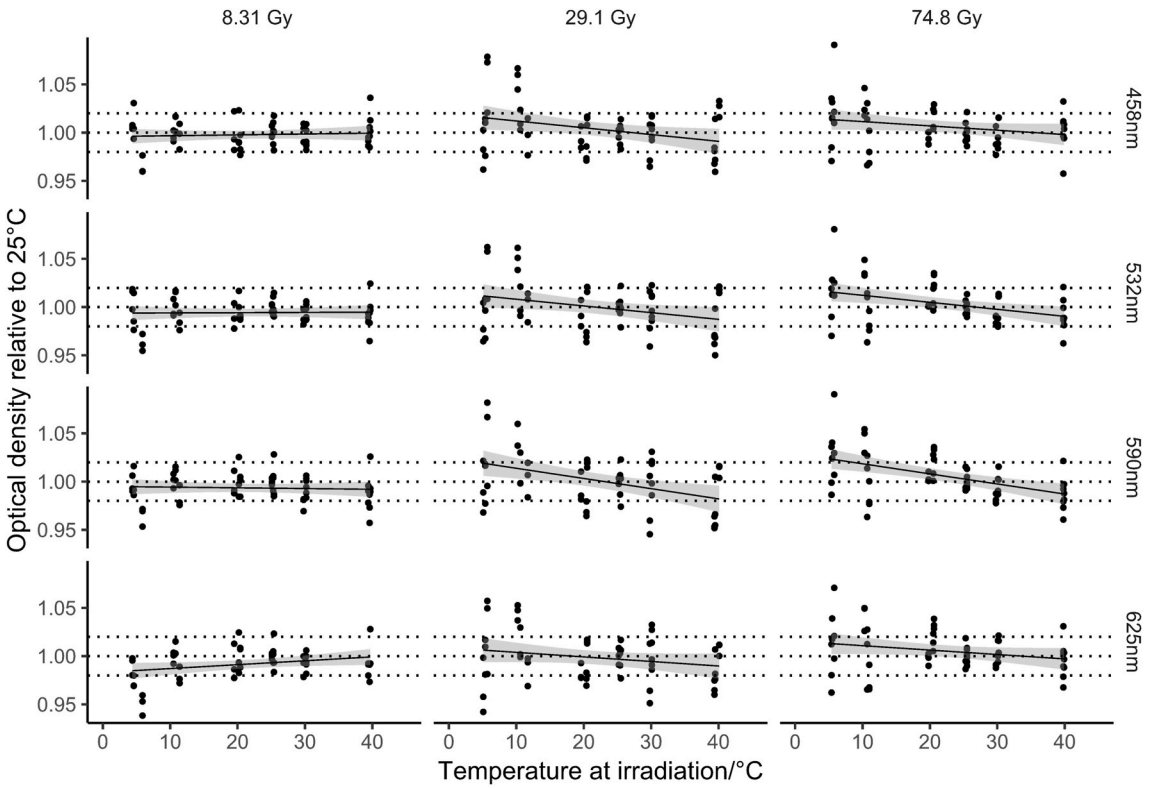

Fig. 3. Response of Gafchromic™ MD-V3 film between 5 and 40 °C relative to 25 °C at three doses and 4 reading wavelengths. Mean response of 3 dosimeters per dose and temperature with linear regression line and 95% confidence interval. Horizontal dotted lines are at 0.98, 1.00 and 1.02.

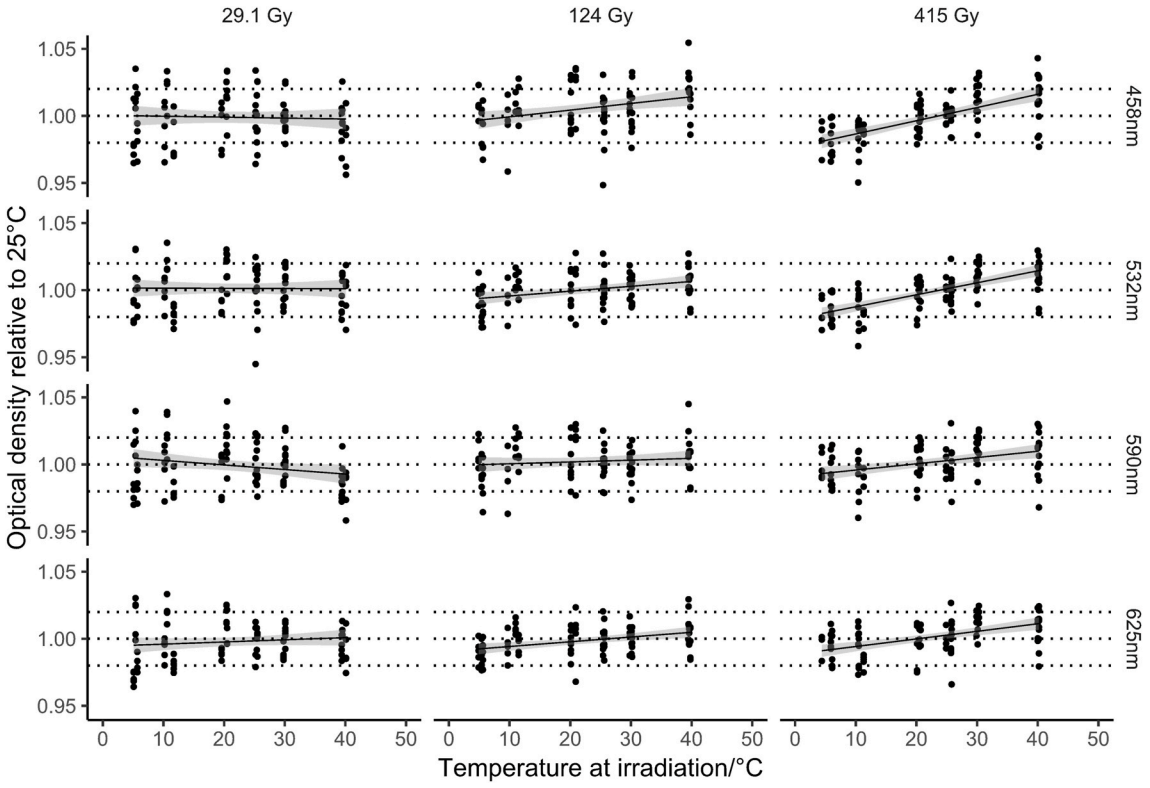

Fig. 4. Response of Gafchromic™ HD-V2 film between 5 and 40 °C relative to 25 °C at three doses and 4 reading wavelengths. Mean response of 5 dosimeters per dose and temperature with linear regression line and 95% confidence interval. Horizontal dotted lines are at 0.98, 1.00 and 1.02.

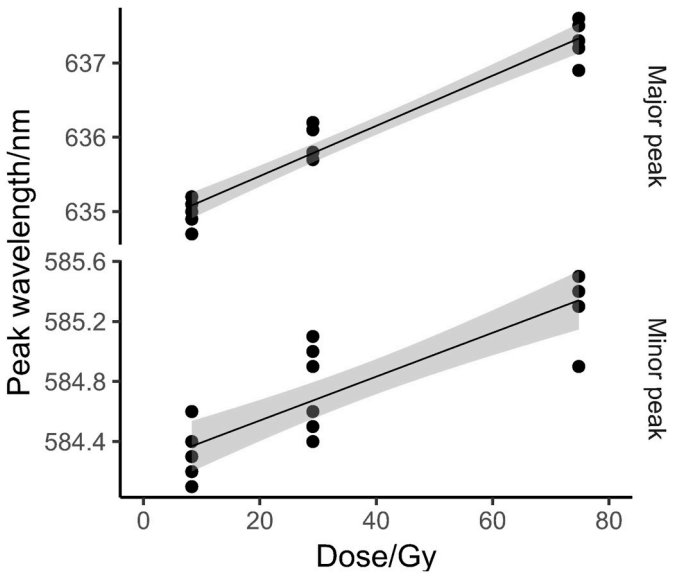

Fig. 5. Influence of dose on the wavelength of the primary and secondary absorption peaks of MD-V3 film.

Gafchromic calibration is typically 3–4% as indicated by the uncertainty budget calculated by the FAO/IAEA workbook (FAO/IAEA, 2022a; 2022b). A typical uncertainty budget would consist of uncertainty from the transfer standard dosimeter (usually alanine for gamma or an ion chamber for X-ray) of 1.6%, from the calibration relationship 1.87%, from lot non-homogeneity 1.95%, and uncertainty in the readout temperature 0.43%, giving a total uncertainty of 3.17% (k = 1) when added in quadrature. Over a moderate temperature range (say 20–30 °C) correction for temperature at time of irradiation is, therefore, unnecessary as the variation due to temperature would introduce an uncertainty of about 0.3% which, when combined with the other uncertainty components increases the total uncertainty by 0.05% to 3.22%. Over the full temperature range of 5–40 °C the increase in the overall uncertainty would be approximately 0.6%, which may generally be acceptable. Where large temperature variations are expected or a lower uncertainty is important it would be necessary to establish a calibration at or near the temperature (±5 °C) that will be used.

The data sheet provided with the HD-V2 film (Ashland, 2021a) includes a plot of the spectrum of the film at various doses but the data sheet with the MD-V3 film (Ashland, 2021b) no longer contains this figure. In previous versions of the data sheet, for older versions of the MD film, a similar figure was included. This plot clearly shows the wavelength of the major and minor peaks shifting towards shorter wavelengths as the dose increases (up to 500 Gy) by about 10 nm, with a note that the active component in HD-V2 is chemically the same as that in MD-V3, but the crystalline form is different and major peak of the MD-V3 film is at about 635 nm. Our observations confirm that the major peak is at about 635 nm for MD-V3, but our observations show the peaks shifting by a much smaller amount (2 nm) towards longer wavelengths at 90 Gy. This smaller shift is at the limit of resolution of the spectrometer used (2 nm). We also observed no measureable change in the peak wavelengths with temperature. The difference in crystalline form of the active layer between HD-V2 and MD-V3 may explain the reversal of the observed effect of temperature at the time of exposure, mostly positive for HD-V2 and mostly negative for MD-V3.

Both the absolute OD and response of the HD-V2 was unexpectedly high (**Supplementary File 1**). Indeed, at 29.1 Gy, where a direct comparison is possible between the MD-V3 and HD-V2 films, the absolute OD and the response were larger in the HD-V2 film than in the MD-V3 film for all replicates and temperatures, except for a few of the 458 nm readings where the HD-V2 and MD-V3 were essentially the same as each other. The very high response of the HD-V2 film reduces the accuracy of the measurements at doses over about 100 Gy as the OD of the film approaches the saturation point of many measuring instruments at wavelengths close to the peaks. Unfortunately, due to the unexpectedly high OD values we were not able to obtain spectra for the 124 or 415 Gy doses with the HD-V2 film to compare directly with the figure in the data sheet.

In conclusion, the new versions of the MD and HD films seem to have been significantly improved in relation to both temperature responses at the time of irradiation as measured with the DoseReader4 at dose rates of about 1 Gy s^{-1} under our experimental conditions and the consistency of the absorption spectrum at different doses. As a result of these improvements the effect of temperature on response and of dose on the spectrum shape can be ignored in most routine use of these films for dose measurement. However, the unexpectedly high response of the HD-V2 film means that the spectral response still needs to be determined and the temperature effects confirmed in a batch with a lower response, as found in other batches of this film.

Conflicts of interest

The authors declare that they have no competing interests.

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Author statement

HY and AP both conceptualized, developed and performed the

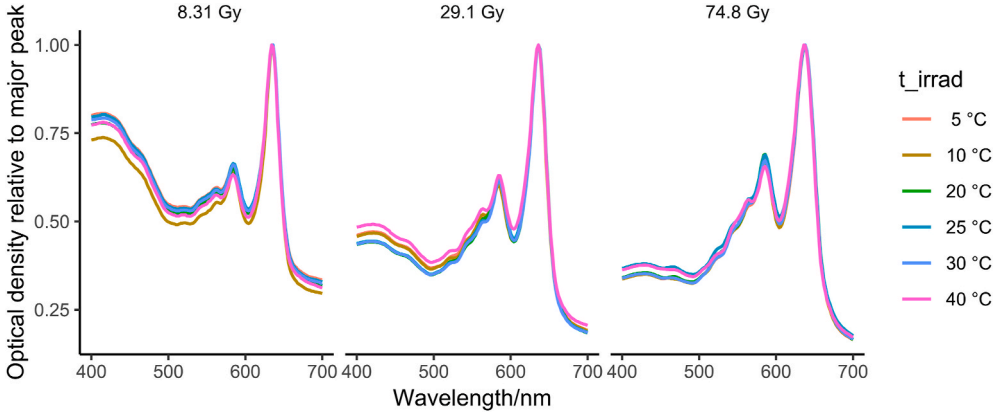

Fig. 6. Influence of temperature on the spectrum of MD-V3 film. The spectra are normalised to the major peak.

experiment protocol. HY and AP shared the writing and editing of the first draft, and later versions of the manuscript. AP performed the statistical analyses and provided the figures.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.radphyschem.2022.110101.

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Parasites & Vectors

RESEARCH Open Access

Identification of critical factors that significantly affect the dose-response in mosquitoes irradiated as pupae

Hanano Yamada[1,2*], Hamidou Maiga[1], Jose Juarez[1], Danilo De Oliveira Carvalho[1], Wadaka Mamai[1], Adel Ali[1], Nanwintoum Severin Bimbile-Somda[1], Andrew Gordon Parker[1], Dongjing Zhang[3] and Jeremy Bouyer[1]

Abstract

Background: The sterile insect technique (SIT) for use against mosquitoes consists of several steps including the production of the target species in large numbers, the separation of males and females, the sterilization of the males, and the packing, transport and release of the sterile males at the target site. The sterility of the males is the basis of the technique; for this, efficient and standardized irradiation methods are needed to ensure that the required level of sterility is reliably and reproducibly achieved. While several reports have found that certain biological factors, handling methods and varying irradiation procedures can alter the level of induced sterility in insects, few studies exist in which the methodologies are adequately described and discussed for the reproductive sterilization of mosquitoes. Numerous irradiation studies on mosquito pupae have resulted in varying levels of sterility. Therefore, we initiated a series of small-scale experiments to first investigate variable parameters that may influence dose-response in mosquito pupae, and secondly, identify those factors that potentially have a significantly large effect and need further attention.

Methods: In this study, we compiled the results of a series of experiments investigating variable parameters such as pupal age (*Aedes aegypti*), pupal size (*Ae. aegypti*), geographical origin of mosquito strains (*Ae. aegypti* and *Ae. albopictus*), exposure methods (in wet *versus* dry conditions, *Ae. albopictus*) and subsequently in low *versus* high oxygen environments [submerged in water (low O_2 (< 5 %)] and in air [high O_2 (~ 21 %)] on the radiosensitivity of male pupae (*Ae. aegypti*, *Ae. albopictus* and *Anopheles arabiensis*).

Results: Results indicate that radiosensitvity of *Ae. aegypti* decreases with increasing pupal age (99% induced sterility in youngest pupae, compared to 93% in oldest pupae), but does not change with differences in pupal size ($P = 0.94$). Differing geographical origin of the same mosquito species did not result in variations in radiosensitivity in *Ae. aegypti* pupae [Brazil, Indonesia, France (La Reunion), Thailand] or *Ae. albopictus* [Italy, France (La Reunion)]. Differences in induced sterility were seen following irradiation of pupae that were in wet *versus* dry conditions, which led to further tests showing significant radioprotective effects of oxygen depletion during irradiation procedures in three tested mosquito species, as seen in other insects.

*Correspondence: h.yamada@iaea.org
[1] Insect Pest Control Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna International Centre, P.O. Box 100, 1400 Vienna, Austria
Full list of author information is available at the end of the article

Conclusions: These findings infer the necessity to further evaluate significant factors and reassess dose-response for mosquitoes with controlled variables to be able to formulate protocols to achieve reliable and reproducible levels of sterility for application in the frame of the SIT.

Keywords: *Aedes aegypti*, *Aedes albopictus*, *Anopheles arabiensis*, Hypoxia, Irradiation, Gammacell, Induced sterility, SIT

Background

In the frame of the sterile insect technique (SIT) [1] and its application towards the management of medically important mosquito species, it is essential to standardize methods for the evaluation of radiation induced sterility in the males produced for releases. 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.

The first step for improvement for the irradiation procedures for mosquitoes is to harmonize materials and methods used and to find the causes for largely varying dose-response data currently found in the literature. Differences in radiosensitivity can be expected between families and even genera within the families of insects [2]; however, such variations in irradiation doses and resulting sterility within the same species beyond a "reasonable range" infers the effects of internal or external variables and these require evaluation and standardization before data on radiosterilizing doses are reliable for a given species.

To date, few publications exist reporting the effects of radiation on mosquito sterility compared to other insects of agricultural importance, and even fewer still adequately describe materials and methods used for the irradiation exposures, or biological variables that were controlled for (or not). No two experiments report the same method or the same result in terms of induced sterility (IS) at a given dose for a particular mosquito species. Important but scattered information, and incomplete descriptions of protocols used for the irradiation of mosquitoes have been described in some publications [3–15]; however, these have shown to be inadequate to reproduce the results consistently amongst research groups around the globe. Balestrino et al. [4] give a useful and detailed summary of past irradiation experiments for a variety of mosquito species, where the authors highlight that the results regarding induced sterility varies significantly amongst these reports. Many miss important factors such as verification of actual doses received by samples due to lack of dosimetry; few describe details of the sample preparation and handling methods, or are lacking information regarding other variable factors such as exact age, irradiation canisters and sample holding containers and handling methods, that may have affected the dose-response. However, there are several parameters that were shown to be important in these studies, and in studies involving other insect species, and these deserve a closer look on their own to assess their impact on dose-response in mosquitoes.

In radiation studies the primary parameter needing reliability and replicability is dose. For research (and operational) 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. The dose rate must, therefore, 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. Therefore, for all studies, the configuration must be standardized to assure that the dose reported, is indeed the true dose received by the sample.

Age has often been shown to have an effect on radiosensitivity. In general, the older the life stage, or the age within the life stages, the more radioresistant the insects become. This is because dividing cells are more susceptible to damage and developing stages of biological tissues are thus generally more sensitive than developed tissue [16]. Older pupae of several species of Tephritid fruit flies [17], Lepidoptera [18, 19] and *Glossina* spp. [20] have also been reported to be more radioresistant as pupal ages increase. Studies in different mosquito species give mixed reports, with different levels of significance given to pupal age as a factor affecting radiosensitivity [4, 10, 13].

In general, the sexes of arthropods have differential responses to irradiation. More often, females are more sensitive to irradiation than males [17, 21, 22], although

there are some exceptions (such as in Glossinidae, males are more radiosensitive than females [23]). The enhanced radiosensitvity in females compared to males is also seen in some mosquito species, such as *An. arabiensis* [24], *Culex pipiens* [14], *Ae. aegypti* [52] and *Ae. albopictus* [4], where females cease to lay eggs altogether at doses of around 60–70, 70, 45 and 30 Gy, respectively.

Genetic differences representing geographical diversity could contribute to slight variations of radiosensitivity within the same species of insect but may not necessarily be the case. Only a few reports exist where such a difference has been described, and explanations for these inherent differences were hypothesized to have developed in response to external factors (such as altitude where the insects were reared) [25, 26]. Indeed, the important variable may have been the differences in size of the insects, resulting from differences in rearing. For mosquito irradiation, it is difficult to tell whether there may be differences in inherent radiosensitivity, as strains irradiated in different institutes, countries and using different protocols cannot be compared effectively. It is also important to keep in mind that different strains for the same species may have a slightly different level of natural sterility. Therefore, it is not useful to compare hatch rates, but rather the corrected hatch rates in reference to the control fertility, in other words, the induced sterility for all experiments assessing the effects on fertility.

Atmospheric conditions during irradiation, particularly differences in oxygen levels, have been shown to have significant impacts on dose-response in insects. Radiation effects are generally reduced in oxygen-poor environments (hypoxia) compared to oxygen-rich environments (normoxia), as radiation induces a chain of oxidative reactions. In the absence of oxygen, the free radicals may combine with hydrogen radicals, reducing the overall impact [27, 28]. The effect of hypoxia on dose-response has been well documented in other insects, particularly in agricultural pests such as the Mediterranean fruit fly (*Ceratitis capitata*), apple maggot (*Rhagoletis pomonella*), oriental fruit moth (*Grapholita molesta*), European corn borer (*Ostrinia nubilalis*) and plum curculio (*Conotrachelus nenuphar*), which were rendered more radioresistant in the absence of oxygen [29–31]. Some reports exist from the 1970s and 80s where atmospheric conditions affected dose-response in mosquitoes. Irradiation of *An. gambiae* (pupal stage), *Culex quinquefaciatus* (pupal and adult stages) and *Ae. aegypti*, all in nitrogen, showed that higher doses were needed to achieve the target sterility [6, 30, 32].

Seeing in other insects that there are numerous variables that have an impact on dose-response, we initiated a series of preliminary studies to investigate these factors with the aim to identify those that may have an important role in the irradiation of mosquito pupae, and to select the factors that may have significant effects in dose-response for further evaluation at a greater scale. This work compiles the results of these preliminary tests which were performed over a period of two years. The experimental methods change between experiments as new information regarding certain parameters became available and understanding of radiation-induced effects increased. It is for this reason that the sample sizes and number of repetitions, or selected doses vary in the different experiments. The long-term aim of this collected work is to acquire sufficient information to develop guidelines for the effective and reliable irradiation of mosquito pupae.

Methods

Mosquito strains

The *Ae. aegypti* strain originated from field collections in Juazeiro (Bahia), Brazil and were transferred to the Insect Pest Control Laboratory (IPCL) of the FAO/IAEA Agriculture and Biotechnology Laboratories, Seibersdorf, Austria from the insectary of Biofabrica Moscamed, Juazeiro, Brazil in 2016.

The *Ae. albopictus* strain originated from field collections in northern Italy and has been maintained under laboratory conditions at the Centro Agricoltura Ambiente, Bologna, Italy. The strain was transferred to the IPCL in 2012. Both the *Ae. albopictus* and *Ae. aegypti* strains have been maintained following the FAO/IAEA guidelines for the routine colony maintenance of *Aedes* mosquitoes [33].

The Dongola strain of *An. arabiensis*, originating from Dongola, Northern State, Sudan, was donated by the Tropical Medical Research Institute, Khartoum, Sudan in 2004 and has been maintained at the IPCL following the FAO/IAEA guidelines for the mass-rearing of *Anopheles* mosquitoes [34].

Four *Ae. aegypti* strains donated from La Reunion (France), Brazil, Thailand and Indonesia and two strains of *Ae. albopictus* from La Reunion (France) and Italy (Rimini), were used in the experiment assessing the effects of differential strain origin on radiosensitivity.

The irradiator

The irradiation device used in these experiments was a Gammacell 220 (Nordion Ltd, Kanata, Ontario, Canada). Over the course of the following experiments, the dose-rate of the source (Co60) decreased from 1.478 to 1.296 Gy/s at the time of the last experiment. The dose rate within the chamber volume varies substantially, with the lowest dose at the top and bottom of the chamber and the highest at the middle periphery (Fig. 1). The overall dose uniformity ratio (DUR) is 1.8, but the dose rate

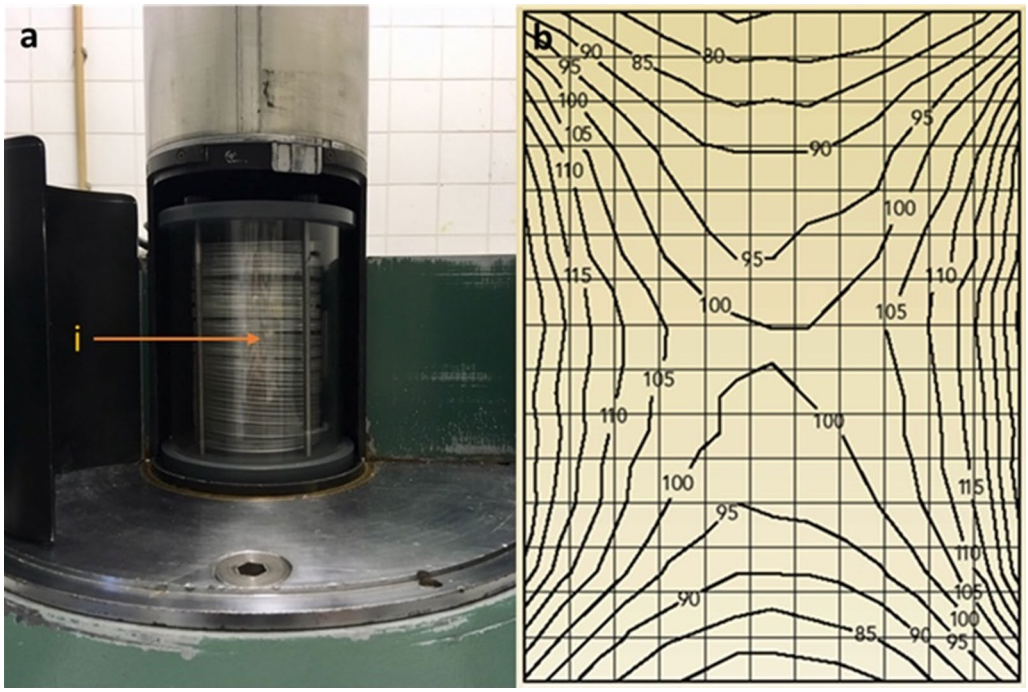

Fig. 1 **a** A Gammacell 220 irradiation chamber containing a mosquito pupae holding canister consisting of stacked Petri dishes (pupae and dosimeters are in a central position). **b** Dose distribution map: a vertical section dose map of a GC220, with doses varying from 75 to 135% of the center dose (DUR = 1.8)

varies least in the middle of the chamber. The custom-made irradiation canister for mosquito pupae irradiation improves the DUR to approximately 1.3 in its interior (Fig. 1). To ensure that pupae samples did not receive significantly varying doses between experiments, the samples were always placed in the same center position on the centremost Petri dish, to further improve the DUR to below 1.1 to ensure consistency.

Dosimetry

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 [35]. 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. A dosimetry system was used in all experiments to verify the dose received by the batches based on Gafchromic HD-V2 and MD-V3 film (Ashland Advanced Materials, Bridgewater NJ, USA) [35]. Three films of either HD film (for doses > 60 Gy) or MD film (for < 60 Gy) were packed in aluminium envelopes (to avoid getting wet) and placed directly above and below the pupae samples. The temperature near the sample and films was measured before and after radiation exposure. Films were read with an optical density reader after 24 h of development.

Effects of strain geographical origin

One variable which is present in all historic and current irradiation studies around the globe is the geographical origin of the mosquito strains. To assess whether this factor could be the reason why different studies by different researchers result in inconsistent results for induced sterility, we compared the dose-response in *Ae. aegypti* and *Ae. albopictus* strains that were donated from different geographical regions. *Aedes aegypti* strains from Brazil, Indonesia, France (La Reunion) and Thailand, and *Ae. albopictus* strains from Italy and France (La Reunion) were assessed.

Approximately 2000 eggs were used for each strain. Larvae were reared according to the "Guidelines for Routine Colony Maintenance of *Aedes* mosquitoes" [33]. Pupae were collected on the sixth day at either 09:00 h, 12:00 h or 15:00 h to obtain > 40-h-old pupae by the time of irradiation. Male and female pupae were separated based on size and confirmed under a microscope. For each strain, female pupae were placed in individual tubes for emergence to ensure virginity and were kept for mating. Male pupae were split into two treatments (control and irradiation) with 50 pupae per treatment, per strain, and with three replicates each. The treatment groups of the 4 strains of *Ae. aegypti*, (and 2 strains of *Ae. albopictus*) were irradiated at 40 Gy in a custom-made Petri

dish containing 4 wells equidistant from the center point, which was placed in the middle of the irradiation canister, with excess water removed, and in the center of the Gammacell chamber (Fig. 1a). For each repetition, the strains were rotated among the wells in the Petri dish.

A total of 40 male pupae were kept for emergence in cages and were mated to virgin females at a 1:1 ratio. Females were offered 3 blood meals (defibrinated porcine blood) to ensure a high overall blood-feeding rate. Females were then separated and placed into individual tubes with distilled water and oviposition paper for egging. Females were allowed to lay eggs over 4 days.

The eggs were then collected, dried and hatched using hatching solution [0.7 l of deionized water, 0.25 g of CM 0001 Nutrient Broth (Oxoid, Hampshire, UK) and 0.05 g of yeast] for 24 h as described by Zheng et al [36]. The number of viable L1 larvae and egg hatch rate was determined under a stereomicroscope and induced sterility calculated.

Effects of pupal age on radiosensitivity in *Ae. aegypti*

The effect of age in *Ae. aegypti* pupae has been assessed and reported for 2 broad age groups (young, 19–23 h; and old, 42–46 h) [37]. To get a better picture of the relationship between pupal age and induced sterility, 5 random age groups (45–50 h; 42–45 h; 26–42 h; 20–24 h; and 10–24 h) were irradiated in Petri dishes, with excess water removed (but pupae still damp), at a fixed dose (40 Gy) and induced sterility was assessed and compared. A diagnostic dose of 40 Gy was selected, aiming to achieve around 90% induced sterility to be able to see increased or decreased effects without arriving at 0% hatching. Age groups were selected according to when 40–50 male pupae (2 times 20 per technical repetition) became available and could be collected. The 2 repetitions involved pupae from the same cohort; however, each rep was irradiated in separate irradiation events and were mated in separate cages, and resulting egg batches were analysed separately. Female pupae were sexed by size using a glass pupal sorter, and then were tubed individually to ensure sex and virginity. Virgin females were added to each cage containing 20 males at a 1:1 ratio and were allowed to mate for 3 days before being offered blood meals on 2 consecutive days to ensure a high overall blood-feeding rate. Oviposition cups lined with germination paper and filled half way up with water were added to the cages 3 days after the first blood meal. Eggs were collected *en masse* and were matured (slow-dried over 4 days) and stored for 10 days before hatching. Hatched larvae were counted, and the remaining unhatched eggs were bleached for *c.* 10 min in a 6% sodium hypochlorite solution to identify any remaining unhatched, fertile eggs. In addition, adult male longevity was followed in both repetitions by removing all dead males every 2–3 days until all males were deceased.

Effects of pupal size on radiosensitivity in *Ae. aegypti*

Aedes aegypti larvae from 2 different cohorts were split and reared in rearing trays at either a very high density (6 larvae/ml) or very low density (1 larva/ml) to obtain small (< 0.900 mm) and large pupae (> 1.100 mm), respectively. Amounts of diet per larva were equal for all trays in both groups. The pupae were collected over a 6-h period to ensure that all pupae were between 24 and 30 h-old at the time of irradiation. The pupae were counted into 4 groups of 30 for the 4 repetitions per size. All groups were irradiated in Petri dishes with excess water removed, at a diagnostic dose of 30 Gy. This dose was chosen as the results for the previous experiment using 40 Gy came close to 99% induced sterility for some treatment groups and full sterility needed to be avoided as a 0% hatching is not comparable. Irradiated males and controls were mated to virgin females (prepared as described in the previous section "Effects of pupal age on radiosensitivity") and the resultant eggs were collected *en masse* and were checked for fertility (also as previously described in the same section).

Effects of sample preparation and ambient conditions during exposure

Aedes albopictus larvae were reared as described in the "strain geographical origin" section. Pupae aged 24–30 h were then divided and prepared to be irradiated in either "dry conditions" or "wet conditions" at 40 Gy. For the "dry" treatment, pupae were dried by placing them onto a paper towel to remove all water. For the "wet" treatment, pupae were placed in a 15 ml falcon tube such that they were swimming/floating in 1 ml of water. The tube containing pupae was held in place in the middle of the irradiation canister (Fig. 1) for irradiation, so that the location of the pupae was the same for both treatments. For each treatment, batches of 100 male pupae were irradiated. Three repetitions were performed for each treatment. Controls consisted of the same number of pupae that were not irradiated. Mating, blood-feeding, egging and egg hatch check were performed as described above in the "strain geographical origin" experiment.

Effects of atmospheric conditions on induced sterility in *Ae. aegypti*, *Ae. albopictus* and *An. arabiensis*

Following the previous experiment assessing handling and sample preparation methods, the ambient conditions were further controlled by inducing a low oxygen environment [using water, which is known to have an O_2 level of 8% and lower at temperatures over 25 °C, (https://www.engineeringtoolbox.com/air-solubility-water-d_639.html)] during irradiation and compared to an environment of air (which is known to have levels of oxygen at around 21%, defined as a normoxic condition).

Sample preparation

Pupae of all 3 species were collected in 4-h windows to ensure uniform pupal age of 40–44 h for both *Aedes* strains, and 20–24 h for *An. arabiensis*. *Aedes* pupae were sexed based on pupal size dimorphism using a glass pupal sorter [38] and sex was verified under a stereomicroscope. Pupae of *An. arabiensis* were sexed visually using a stereomicroscope. Males were kept for treatment and females were placed in individual tubes for emergence to ensure sex and virginity for later mating. To establish a low oxygen environment, male pupae were counted into batches of 160 and were placed in 1.5 ml Eppendorf tubes containing water. This pupal density was selected according to observations by D. Zhang in which pupae at this density, submerged in water showed a decreased radiosensitivity compared to other reports on irradiation of the same strain. For the normoxic conditions, pupae were placed in the center (2 cm diameter ring made with a hot-melt adhesive) of 100 × 15 mm standard Petri dishes for irradiation. Excess water was removed so that the pupae were surrounded by air. The Eppendorf tubes were closed 30 min prior to irradiation treatment to establish hypoxia (low oxygen environment) by allowing depletion of oxygen by respiration through the cuticles of the contained pupae, as was seen by Zhang (unpublished data). Both the pupae in hypoxic and normoxic treatment groups were irradiated at the same time to ensure equal dose. Four biological repetitions were performed for both *Ae. aegypti* and *Ae. albopictus*, and 3 repetitions for *An. arabiensis*, each with 3 technical repetitions.

Irradiation

Radiation treatments and controls were performed in either hypoxic or normoxic conditions. A diagnostic dose was selected according to the expected dose required to induce less than 100% sterility in each strain and each treatment: 70, 35 and 95 Gy for *Ae. aegypti*, *Ae. albopictus* and *An. arabiensis*, respectively.

Assessment of induced sterility

Following irradiation, 50 males were randomly selected from each treatment group and were placed in a 15 × 15 × 15 cm BugDorm cage (MegaView Science Co. Ltd., Taichung, Taiwan) for emergence. Fifty virgin females prepared as described above (and of the same age) were added to each cage and were allowed to mate for 3 days. All batches were provided with 2 blood meals on consecutive days to increase overall blood-feeding rates following the mating period. Oviposition cups containing water and egg papers (germination paper) were added to each cage for egg collection *en masse* following routine rearing protocols [33]. Egg papers from *Ae. aegypti* and *Ae. albopictus* were collected, matured (slow-dried over 4 days) and stored for 10 days before hatching. Hatch rates were determined under the stereomicroscope. Unhatched eggs were bleached for *c.* 10 min in a 6% sodium hypochlorite solution to identify any remaining unhatched, but fertile eggs. The *An. arabiensis* eggs were collected and hatched the same day. The total number of eggs and the number of L1 larvae were counted for each treatment group to derive the hatch rate. The obtained hatch rate was further verified by counting the number of hatched and un-hatched eggs using a stereomicroscope and the induced sterility was calculated.

Statistics

The residual fertility (RF) was calculated as a percentage of the control fertility of each treatment group ($RF = HR_{tx}/HR_c \times 100$), where HR_{tx} is the hatch rate of the treatment (tx) group, and HR_c is the hatch rate of the control (c) group. Induced sterility (IS) was calculated by subtracting the RF from 100%.

Statistical analyses were performed using Microsoft Excel (v.16.0, Microsoft, Redmond, WA, USA), GraphPad Prism v.5.0 (Graphpad Software, La Jolla, CA, USA; http://www.graphpad.com) and R v.3.5.2 [39].

The effect of geographical origin on induced sterility was analyzed using a Gaussian linear mixed-effects model fit by maximum likelihood, with strain/country as fixed effect and repetitions as a random effect.

A Pearson's correlation coefficient was used to detect the linear correlation of pupal age and the induced sterility. ANOVA was performed to test the effect of pupal age on induced sterility followed by Tukey's multiple comparisons of means to compare each pair of age group. The longevity of males from all age groups was analyzed using Kaplan–Meier survival analyses. The log-rank (Mantel–Cox) test was used to compare the level of survival between different age groups. To account for the multiplicity of comparisons the Bonferroni correction method was applied for each pair of age group. For this test, alpha levels were corrected to $P < 0.0033$.

A Gaussian linear mixed-effects model fit by maximum likelihood, with pupal size as fixed effect and repetitions as a random effect was used to analyze the effect of pupal size on induced sterility. The same analysis was used to test the effects of sample preparation and ambient conditions during exposure on induced sterility.

A binomial linear mixed effect models were used to analyze the impact of hypoxia on the hatching rate (R). The treatment regimens for irradiation were then used as fixed effects and the repetitions as random effects. The significance of fixed effects was tested using the likelihood ratio test [40, 41]

The best model in all analyses was selected based on the lowest corrected Akaike information criterion

(AICc), and the significance of fixed effects was tested using the likelihood ratio test. All significant differences are based on $P < 0.05$.

Results

Dosimetry

The dosimetry confirmed that all doses received lay within the 5% confidence interval of the calibration.

Strain geographical origin

There was no significant difference seen in the induced sterility between the 4 strains of *Ae. aegypti* [originating from Brazil, Indonesia, France (La Reunion) and Thailand] [$P > .05$, for all comparisons ($P = 0.1873$, 0.4215, and 0.3919), Fig. 2], nor between the two strains of *Ae. albopictus* (originating from Italy and France (La Reunion); $P = 0.14$, Fig. 3), following irradiation under controlled conditions.

Effects of age on radiosensitivity and longevity in *Ae. aegypti*

As expected, there was a strong negative correlation between pupal age and radiosensitivity (in terms of resulting induced sterility) (Table 1) with the youngest pupae the most sensitive to irradiation and resistance increasing as the pupal age increases (using median age per group: $R^2 = -0.9513$; youngest age per group: $R^2 = -0.9593$; oldest age per group: $R^2 = -0.8983$).

The radiosensitivity was significantly impacted by pupal age ($F_{(5, 7)} = 34218$, $P < 0.0001$). When different age group were compared, old pupae (45–50 h) were more resistant to irradiation compared to all other pupal ages tested in this experiment [$P < 0.05$ for all comparisons ($P < 0.0001$, $P = 0.0082$, 0.00057 and 0.00022, $P < 0.0001$]). Pupae older than 26 h were also more resistant than that of age below 24 h ($P = 0.0018$ and 0.0061).

Results relating to the longevity of adults following irradiation at a fixed dose at pupal stages with varying age are summarized in Table 2. The mean longevity was significantly reduced only for the pupae irradiated at the youngest age (10–24 h; $P < 0.0001$). The number of days for the population to reduce to 50% was only significantly reduced in the youngest age group ($P < 0.0001$), who were generally the poorest survivors amongst the treatment groups.

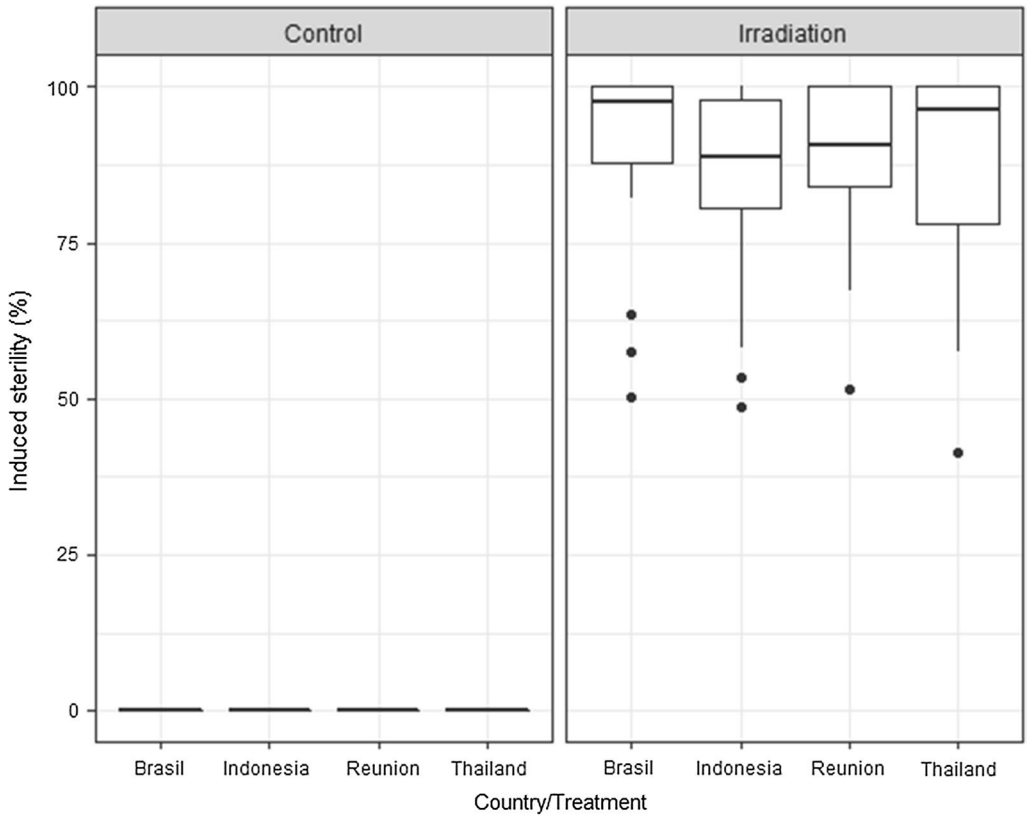

Fig. 2 Comparison of radiosensitivity *Ae. aegypti* strains from different geographical origins. No difference was observed following irradiation at 40 Gy. The box plot shows the median and upper and lower quartiles

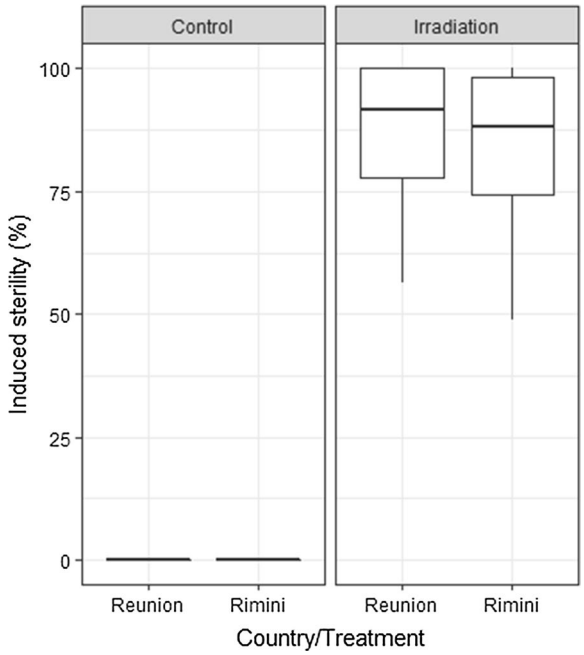

Fig. 3 Comparison of radiosensitivity *Ae. albopictus* strains from different geographical origins. No difference was observed following irradiation at 30 Gy. The boxplot shows the median and upper and lower quartiles

Effects of pupal size on radiosensitivity in *Ae. aegypti*

Following irradiation at 30 Gy, the variation amongst samples was quite high. However, the mean induced sterility in small and large pupae was 76.5 ± 6.5% and 75.9 ± 4.6% (mean ± SE) respectively (Fig. 4). No difference in radiosensitivity was observed between the samples of small and large pupae ($P = 0.94$).

Effects of sample preparation and ambient conditions during exposure

Significant differences were seen in dose-response (and resulting induced sterility) following irradiation of the *Ae. albopictus* pupae in controlled conditions, but with differing sample preparation methods ($t_{(104)} = -2.87$, $P = 0.0049$) (Fig. 5). In addition to this, more variation was observed amongst individual males irradiated in water (wet treatment), although outliers were present in both treatment groups (Fig. 4).

Effects of hypoxia on induced sterility in *Ae. aegypti*, *Ae. albopictus* and *An. arabiensis*

In all three species, irradiation at the pupal stage significantly reduced the hatch rate ($P < 0.0001$). Moreover, hypoxic conditions showed an attenuation of effects and overall levels of sterility induced (Table 3; $P < 0.0001$). A 3.8-fold increase in residual fertility was seen in hypoxic *versus* normoxic conditions in *Ae. aegypti* after a dose of 70 Gy, with similar trends found for *Ae. albopictus* (at 35 Gy) and *An. arabiensis* (at 95 Gy) with a 2.3 and 3.0-fold increase, respectively.

When hatch rates were corrected by the natural fertility rate measured in the control group [42] to calculate the induced sterility (IS), the significant differences were equally pronounced between the two treatments (Table 3).

There was no correlation of the total number of eggs produced per cage and the treatment for any of the three species, indicating that it is unlikely that different atmospheric treatments on males have any effect on overall fecundity in their female mates. Control hatch rates averaged 85%, 67% and 70% for *Ae. aegypti*, *Ae. albopictus* and *An. arabiensis*, respectively. The results are summarized in Table 3.

Discussion

For the experiments singling out factors that could affect the dose-response in mosquito pupae, it was important to ensure that other factors that could influence the results were minimized, if not completely eliminated. The primary factor that requires standardization is dose. Therefore, before the series of experiments was initiated,

Table 1 Induced sterility in five varying age groups of *Ae. aegypti* pupae following irradiation at 40 Gy

	Control	Pupal age				
		45–50 h	42–45 h	26–42 h	20–24 h	10–24 h
No. of reps	3	2	2	2	2	2
Gy	0	40	40	40	40	40
n	1942	507	799	1203	793	1262
avg (HR)	0.80	0.05	0.04	0.03	0.02	0.01
SE	0.01	0.00	0.00	0.00	0.00	0.00
IS	0.00[a]	93.45[b]	95.37[c]	96.44[cd]	96.91[d]	98.93[e]

Abbreviations: *n*, total no. of eggs; avg HR, average hatch rate; reps, repetitions; SE, standard error; IS, induced sterility

Note: Values followed by different superscript letters are significantly different ($P < 0.05$)

Table 2 Mean longevity and mean number of days required for the population to be reduced to 50 or 0% of the starting population size (*P* threshold value is 0.0033 when comparing all groups)

Pupal age	Days to population reduction			Mean longevity (days)
	50%	90%	100%	(mean ± SE)
Control	23.0	32.6	38.0	19.57 ± 3.0[a]
45–50 h	27.3	36.5	38.0	22.26 ± 3.3[a]
42–45 h	25.5	34.5	38.0	25.40 ± 1.8[a]
26–42 h	23.3	37.2	38.0	22.56 ± 2.3[a]
20–24 h	19.0	34.8	38.0	21.29 ± 2.9[a]
10–24 h	11.0	24.4	28.0	12.81 ± 1.9[b*]

Note: Values followed by different superscript letters are significantly different from each other [log-rank (Mantel-Cox) test *$P < 0.0033$]

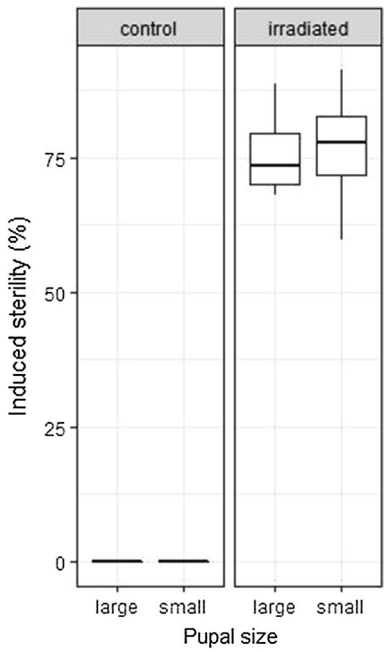

Fig. 4 Effects of pupal size on dose-response in *Ae. aegypti* male pupae. No difference was observed following irradiation at 40 Gy. The boxplot shows the median and upper and lower quartiles

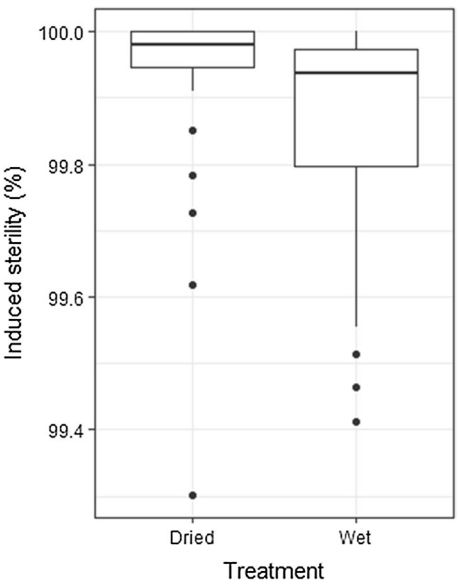

Fig. 5 Effects of sample preparation and ambient conditions during exposure. A significant difference was detected between the dry and wet treatments ($P < 0.005$). The box plot shows the median and upper and lower quartiles. Dots indicate outlying data points

the irradiator (and its irradiation chamber) as well as the dosimetry were characterized and calibrated, and samples were always placed in the same position within the irradiation chamber. 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) [2]. 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). For the irradiation of mosquito pupae in the Gammacell (Co-60 source), a PMMA (polymethylmethacrylate) tube served as the canister wall, and also as the appropriate build-up material during exposures as its interaction with ionizing radiation is near equivalent to water (as are pupae and biological tissue in general). A thickness of the PMMA of 4 mm is sufficient to achieve an electron equilibrium in the chamber and sample, and for this reason it is recommended for routine irradiation work.

In general, *Anopheles* and *Culex* spp. seem to be more radioresistant than *Aedes* spp. In existing literature, doses (gamma-ray) required for > 99% sterility in males of *Anopheles* spp (including *An. albimanus*, *An. arabiensis*, *An. gambiae*, *An. pharaoensis* and *An. stephensi*) are found to range from ~ 80 to 120 Gy [3, 7, 10, 11, 13]. *Culex* spp. males (*Cx. pipiens molestus*, *Cx. quinquefasciatus*, *Cx. tarslis*) have been reported to require doses around 75–150 Gy for full sterility [12, 15, 32], whereas doses reported for *Aedes* spp. (*Ae. aegypti* and *Ae. albopictus*) are generally lower, ranging from 40 to 90 Gy [4, 41, 45]. In these various studies, pupal ages during exposures

Table 3 Induced sterility in adults following irradiation at pupal stage in either normoxic or hypoxic conditions, compared to unirradiated controls

Strain	Treatment	No. of true reps (tech. reps)	Dose (Gy)	HR ± SD (%)	IS (%)	SE (IS)	SD (IS)
Ae. aegypti	Control	4 (3)	0	85.41 ± 0.09	0.00[a]	0.0253	na
	Normoxia	4 (3)	70	1.49 ± 0.01	98.25[b]	0.0021	0.04
	Hypoxia	4 (3)	70	5.67 ± 0.01	93.35[c]	0.0043	0.04
Ae. albopictus	Control	4 (3)	0	66.59 ± 5.56	0.00[a]	2.7778	na
	Normoxia	4 (3)	35	14.32 ± 9.12	78.49[b]	4.5574	0.04
	Hypoxia	4 (3)	35	32.30 ± 5.09	51.49[c]	2.5438	0.06
An. arabiensis	Control	3 (3)	0	70.32 ± 2.38	0.00[a]	0.8131	na
	Normoxia	3 (3)	95	2.29 ± 0.59	96.74[b]	0.3415	0.01
	Hypoxia	3 (3)	95	6.76 ± 1.41	90.39[c]	1.3763	0.01

Note: Values followed by different superscript letters are significantly different $P < 0.0001$. *Abbreviations*: HR, hatch rate; IS, induced sterility; reps, repetitions; SD, standard deviation; SE, standard error; na, not applicable

are not always the same, and other external factors may also widen the dose range reported, such as rearing and handling methods, or nutritional status of the insects. In our studies, the doses required for 99% induced sterility or more are within the ranges reported above, albeit closer to the upper limits of these ranges. This may be due to the fact that we generally used oldest age ranges for the pupae, and the irradiator and its source (activity), rearing methods, larval diets, etc, are of course unique. Although there were differences in dose-response for each of the three species evaluated here, the geographical origin of the strains of both *Aedes* species did not significantly affect their radiosensitivity. Induced sterility in *Ae. albopictus* collected from different localities in northern Italy was also directly compared by Balestrino et al. [4] but these were also found to be similar.

It has been demonstrated in mosquitoes and other insects that radioresistance increases with age [4, 43, 44]. It is therefore important to control for sample age when characterizing a mosquito's dose-response and to know the variation expected within an irradiated sample when the age amongst individuals varies. When choosing an appropriate dose to attain a target sterility level, the dose should be selected according to the radiosensitivity of the oldest (and thus most resistant) pupae to ensure that the rest of the pupae will achieve at least this level of sterility. Little effect of pupal age was found for three age groups of *Ae. albopictus* pupae in a study by Balestrino et al [4]. Conversely, it has been shown here in *Ae. aegypti*, that the correlation of pupal age and radiosensitivity is quite linear, and differences in age can alter the reported dose-response result in different assessments, despite the implementation of same protocols, irradiation device and mosquito strain. It is therefore necessary to accommodate the age-related radioresistance with higher irradiation doses to achieve the target sterility. It has been shown in other insects that in general, irradiation at later ages, or later life stages increases biological quality of the adult males [3, 30, 45]. However, Helinski & Knols [46] observed that when *An. arabiensis* pupae were irradiated with a fully sterilizing dose, no difference between young and old pupae was observed in terms of adult male competitiveness. Additionally, no noticeable effect on *Ae. aegypti* male competitiveness was observed following irradiation at doses up to 100 Gy when early (19–23 h) and late (42–46 h) pupae were irradiated [37]. In this study, a broader age range was assessed, and a difference in radiosensitivity was detected at 40 Gy (a non-fully-sterilizing dose). Although the resulting male adult competitiveneness was not included in this study, the male longevity data showed that pupae irradiated when older than 24 hours survived equally well compared to the controls. Pupae aged 24 hours or less survived poorly compared to the other groups. However, it is important to note that the dose used was a diagnostic dose of 40 Gy and may not induce the same trend as would be the case for higher doses. This experiment still showed that pupal age during exposures can impact the resulting sterility as well as longevity, and therefore consistency in sample age is essential for consistent sterility results.

Although in general larger particles absorb more radiation (kJ/kg), and so should be more radiosensitive, in mosquito pupae (*Ae. aegypti*) there were no observable effects of pupal size on dose-response. Most likely, the differences in mosquito pupae size are generally too small to create an effect. However, small differences in preparation and handling methods of the pupae samples can affects dose-response. Pupae placed in water are less sensitive to irradiation than pupae that are placed in a dry environment, i.e. where all water has been removed. Furthermore, there was more variation in induced sterility within the wet sample group compared to the dry

treatment samples, where induced sterility was more uniform. One reason may be that pupae swimming in water are partially submerged, and oxygen levels in the water are lower than in air (~8% at insectary water temperatures, compared to 21% in air; https://www.engineeringtoolbox.com/air-solubility-water-d_639.html), partially protecting the pupae from irradiation effects [53]. In which case, irradiation on the surface of water cannot ensure a standard exposure of all pupae in a given sample. To further test the hypothesis that pupae submerged in water are protected due to decreased in oxygen saturation, as was observed in the mass-irradiation for *Ae. albopictus* pupae (in water) in the Wolbaki mosquito mass-rearing facility in Guangzhou, China (Zhang et al., unpublished data), it was decided to further investigate this factor in a follow-up experiment where the oxygen levels were the focus.

In this next experiment, submerging pupae in water in a closed container aimed to create a low oxygen environment [hypoxic, in this case referring to a starting DO (dissolved oxygen) level of ~8%]. We suspect that this initial DO further decreases as pupae continue to absorb O_2, presumably by cuticular respiration [54, 55] (Zhang et al., personal communication). We hypothesize that it is the reduced oxygen saturation in the sample environment during irradiation that reduces the irradiation effects, resulting in less sterility induced in the pupae. Hypoxia is known to have a radioprotective effect. This has also been shown in other insect species, although most reports described the irradiation exposure of insect samples in nitrogen. The radioprotective effect of hypoxia in insects requires higher doses to achieve lethality and full sterilization [47]. A low oxygen environment induces damages at the cell level, which leads to an increase in the antioxidant defence mechanisms and enhances the antioxidant capacity during hypoxia to prepare the cell for reoxygenation and minimize oxidative stress [48]. This cellular response that prepares cells for reoxygenation may be one of the crucial factors contributing to the increase in radioresistance. This effect was demonstrated by a study conducted with moths, which compared different developmental stages in hypoxia and normoxia. They showed that for all stages, the individuals that were subjected to hypoxia showed better survival compared to those kept in normoxic conditions [49, 50]. To further verify our hypotheses, that pupae absorb oxygen when submerged in water, thus creating an oxygen-poor (or hypoxic) environment, and that this reduced level of DO has a radioprotective effect in irradiation, we have initiated further studies in the pupae of three species of mosquitoes.

In the present study, we found that the fecundity of the females mated to males irradiated in either atmospheric condition was not affected, and no trend was apparent throughout the repetitions or the strains. This was expected as all females were likely mated and egg production generally relies on the biology of the female. However, we found significant reductions in induced sterility in males irradiated in water in all three species tested. This was most pronounced in *Ae. albopictus* following exposure at 35 Gy with more than a 30% reduction, followed by *An. arabiensis* and *Ae. aegypti* with a 5 and 6.6% reduction in induced sterility, respectively. The large difference seen in *Ae. albopictus* may be due to the overall low hatch rate in this particular cohort. The strain is usually reported to have a hatch rate of over 75% [4, 51]. On the contrary to expectations and past experience, residual fertility was high following exposure at 35 Gy. The reduced hatch in controls *versus* the high fertility in treatment groups may be due to poor storage condition of the eggs, or due to *ad hoc* diapausing behaviour of the females and needs to be further investigated. However, the differential response to irradiation in the two atmospheric conditions was clearly demonstrated and has been reproducible in further studies (data not shown). This effect has also been observed in the operational setting at the Wolbaki mosquito facility. Pupae of *Ae. albopictus* are mass-irradiated (up to 250,000 pupae) in a container filled with water. It was noticed that a higher dose was required to achieve the target sterility levels than was needed for routine, small-scale irradiation in air (D. Zhang, unpublished data). This shows the necessity to reassess dose-rate and dose by dosimetry, and dose-response behaviour in mosquito pupae following any changes in protocols.

Further studies are needed to assess the impact of the radioprotectant properties of hypoxia during irradiation on resulting male biological fitness and to evaluate the advantages and disadvantages of irradiating in water (pupae), nitrogen (adults) or in air with respect to operational practicality and adult sterile male mating propensity and competitiveness in the field.

Conclusions

Differences in pupal age and changing handling methods and atmospheric conditions have major impacts on the resulting sterility reported for mosquitoes. This demonstrates the need for further clarification in larger-scale trials for the development of standardized methods for treatment prior to- and during radiation exposure with the aim to reliably sterilize male mosquitoes, and to produce competitive males for field releases to control populations of these important disease vectors. Next, we aim to re-evaluate full dose-response curves for the three species of mosquitoes following irradiation in hypoxic *versus* normoxic conditions.

Abbreviations
DO: dissolved oxygen; FAO: Food and Agriculture Organization; HR: hatch rate; IAEA: International Atomic Energy Agency; IPCL: Insect Pest Control Laboratory; IS: induced sterility; NIST: National Institute of Standards and Technology; PMMA: polymethylmethacrylate; RF: residual fertility; SIT: sterile insect technique.

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Authors' contributions
HM and JJ conceptualized the design of experiments evaluating the effects of strain geographical origin and sample handling and preparation methods. HY, HM, JJ, DC and WM and carried out the experiments. NSBS, WM and AA provided assistance to the insect rearing and experimental work and preparations. DC, HM, AP, DZ and JB contributed to the experimental designs and to the later versions of the manuscript. JB and HM carried out the statistical analyses. JB supervised and supported the project. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the present study including all dosimetry reports are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1] Insect Pest Control Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna International Centre, P.O. Box 100, 1400 Vienna, Austria. [2] Institute for Insectbiotechnology, Justus-Liebig-University Gießen, Winchester Str. 2, 35394 Gießen, Germany. [3] Key Laboratory of Tropical Disease Control of the Ministry of Education, Sun Yat-sen University - Michigan State University Joint Center of Vector Control for Tropical Diseases, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510080, Guangdong, China.

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The role of oxygen depletion and subsequent radioprotective effects during irradiation of mosquito pupae in water

Hanano Yamada[1,4*], Hamidou Maiga[1], Nanwintoum Severin Bimbile-Somda[1], Danilo O. Carvalho[1], Wadaka Mamai[1], Carina Kraupa[1], Andrew G. Parker[1], Aiman Abrahim[2], Georg Weltin[3], Thomas Wallner[1], Marc F. Schetelig[4], Carlos Caceres[1] and Jeremy Bouyer[1]

Abstract

Background: Radiation induced sterility is the basis of the Sterile Insect Technique, by which a target insect pest population is suppressed by releasing artificially reared sterile males of the pest species in overflooding numbers over a target site. In order for the sterile males to be of high biological quality, effective standard irradiation protocols are required. Following studies investigating the effects of mosquito pupae irradiation in water *versus* in air, there is a need to investigate the oxy-regulatory behavior of mosquito pupae in water to better understand the consequences of irradiation in hypoxic versus normoxic conditions.

Methods: Pupae of *Aedes aegypti*, *Ae. albopictus*, and *Anopheles arabiensis* were submerged in water inside air-tight 2 ml glass vials at a density of 100 pupae/ml and the dissolved oxygen (DO) levels in the water were measured and plotted over time. In addition, male pupae of *Ae. aegypti* (aged 40–44 h), *Ae. albopictus* (aged 40–44 h) and *An. arabiensis* (aged 20–24 h) were irradiated in a gammacell220 at increasing doses in either hypoxic (water with < 0.5% O_2 content) or normoxic (in air) conditions. The males were then mated to virgin females and resulting eggs were checked for induced sterility.

Results: All three species depleted the water of DO to levels under 0.5% within 30 minutes, with *An. arabiensis* consuming oxygen the fastest at under 10 minutes. Following irradiation, the protective effect of hypoxia was observed across species and doses ($P < 0.0001$), increasing at higher doses. This effect was most pronounced in *An. arabiensis*.

Conclusions: The consumption of dissolved oxygen by pupae submerged in water was significantly different between species, indicating that their oxy-regulatory capacity seems to have possibly evolved according to their preferred breeding site characteristics. This needs to be considered when sterilizing male mosquitoes at pupal stage in water. Depending on species, their DO consumption rates and their density, irradiation doses needed to achieve full sterility may vary significantly. Further assessments are required to ascertain optimal conditions in terms of ambient atmosphere during pupal irradiation to produce competitive sterile males, and temperature and density dependent effects are expected.

Keywords: *Aedes aegypti*, *Aedes albopictus*, *Anopheles arabiensis*, Dissolved oxygen, Pupa respiration, Hypoxia, Irradiation, Gammacell, Induced sterility, SIT

*Correspondence: h.yamada@iaea.org; 28429h.yamada@iaea.org
[1] Insect Pest Control Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna International Centre, P.O. Box 100, 1400 Vienna, Austria
Full list of author information is available at the end of the article

Background

The management of mosquitoes (and other insect pests) using the sterile insect technique (SIT) relies on the successful mating of factory reared, sexually sterilized males that are released into the target area in overflooding numbers with wild females [1]. The SIT has been implemented as a part of Area-Wide Integrated Pest Management (AW-IPM) programmes for a variety of insect pests over the past 60 years, and has also been assessed for the suppression of some mosquito species, notably *Anopheles albimanus* [2, 3] and several *Culex* species [4–9], in the 1970s and 1980s. Only recently, the development of the SIT for the management of other mosquito species has begun to gain more focus in response to outbreaks of chikungunya in Europe, global increasing incidences of dengue fever, the re-emergence of yellow fever and the recent emergence of the Zika virus, which are all transmitted by *Aedes aegypti* and/or *Aedes albopictus* [10–17].

There are several options to achieve sterility in insects [18] including transgenic approaches [19], use of *Wolbachia* (i.e. for mating incompatibility) [20], use of chemicals and use of ionizing radiation [21]. Historically, a variety of chemosterilants have been used for the reproductive sterilization of male mosquitoes with varying success and suitability for larger scale SIT programs [3, 9, 11, 22]. However, the evaluation of sterilizing male mosquitoes by irradiation has suggested that this is, to date, the most practical (and environmentally friendly) way to induce sterility, especially at large scale [23]. Some publications reporting the dose-response of mosquitoes are available [23–26], and more recently, more work has been done regarding the effects of irradiation on mosquito fertility, longevity, flight ability and mating competitiveness [27–33] providing a good basis for the SIT package for mosquitoes. However, a more detailed look into other factors affecting radiation induced sterility, including handling methods, irradiation device and source, and intrinsic biological factors of the mosquito, are essential for the development and formulation of standard protocols for the reliable and reproducible induction of sterility.

One of the main factors known to change the radiosensitivity in insects is the atmospheric conditions that they are subjected to before, and during radiation exposure. In Mediterranean fruit flies (*Ceratitis capitata*), it has been shown that the conditioning of the pupae pre-irradiation by inducing hypoxic conditions affected the radiosensitivity of the flies and reduced the "oxygen effects" during irradiation, resulting in more competitive sterile males [34]. Irradiating agricultural commodities in the context of phytosanitary treatments in reduced levels of oxygen rendered other insect species such as the apple maggot (*Rhagoletis pomonella*), oriental fruit moths (*Grapholita molesta*), European corn borer (*Ostrinia nubilalis*), and plum curculio (*Conotrachelus nenuphar*) more resistant to the radiation treatment and residual fertility in some cases increased up to 20-fold *versus* those irradiated in air [35–37].

Whilst protocols for the irradiation of agricultural pests have become very standardized following decades of experience in the context of the SIT and phytosanitary treatments [38], mosquito irradiation protocols around the world are still very diverse, making reliability and reproducibility difficult. As male pupae sterilization is shifting from small sample sizes in Petri dishes for experimental work to bulk exposures of thousands or hundreds of thousands for field releases, handling and exposure protocols are changing for improved efficiency and practicality. A change in dose required for the complete sterilization of *Ae. albopictus* pupae was seen following bulk irradiation in water [39, 40], suggesting that the respiration or transpiration of the pupae reduces dissolved oxygen levels in the water, thus creating a hypoxic environment. However, little has been demonstrated in mosquito pupae in terms of their mechanism(s) of oxygen usage and their behavior when fully submerged under water. Generally, mosquito pupae seem to be bimodal breathers, obtaining oxygen *via* their respiratory trumpets directly from the air when they float on the water surface [41], and *via* diffusion of dissolved oxygen from the water through their cuticle when submerged [42, 43]. Aquatic insects often have a thin permeable integument that enables cuticular respiration and thus the diffusion of gases while under water.

To evaluate the oxygen consumption characteristics of mosquito pupae for irradiation purposes, we assessed dissolved oxygen depletion by pupae in small artificial aqueous environments to ensure the time required to achieve hypoxic conditions prior to evaluating the impact of hypoxia on the dose-response to irradiation for *Ae. aegypti*, *Ae. albopictus* and *An. arabiensis* male pupae. The resulting data aim to improve protocols for the SIT against these mosquito species.

Methods

Mosquito strains

The *Ae. albopictus* strain used for the experiment originated from field collections in northern Italy and has been maintained at the Centro Agricoltura Ambiente, Bologna, Italy. The strain was transferred to the Insect Pest Control Laboratory (IPCL) of the FAO/IAEA Agriculture & Biotechnology Laboratories, Seibersdorf, Austria in 2010.

The *Ae. aegypti* strain originated from field collections in Juazeiro (Bahia), Brazil and was transferred to the ICPL from the insectary of Biofabrica Moscamed, Juazeiro, Brazil, in 2016. Both the *Aedes* strains have been maintained following the "Guidelines for Routine Colony Maintenance of *Aedes* mosquitoes" [44].

The Dongola strain of *An. arabiensis*, originating from Dongola, Northern State, Sudan, was donated by the Tropical Medical Research Institute, Khartoum, Sudan, in 2010 and has been maintained at the IPCL following the anopheline mass-rearing guidelines [45].

Measurement of oxygen depletion by pupae in water

Pupae from the first day of pupation (and therefore mostly male) of each mosquito species were counted into batches of 200 and were transferred into 2 ml autosampler vials (Merck KGaA, Darmstadt, Germany) which were then topped up to the rim with water to ensure that there is no air bubble inside the vial when closed. The water and ambient temperature were measured at 25 °C. The temperature and dissolved oxygen (DO) in the water was measured with a DO-166MT-1 micro dissolved Oxygen electrode (Lazar Research Laboratories, Inc., Los Angeles, CA, USA) which was inserted into the sample through the hole in the cap of the autosampler vial and was fixed by a plastic valve and blu tac adhesive putty. The electrode was attached to an amplifier and then to a Microcomputer based pH/mV/Temp portable meter (Jenco Model 6230N, Jenco Instruments Inc., San Diego, CA, USA). The electrode was calibrated prior to each experiment using a two-point calibration: in air (21% oxygen, and in water which was bubbled with Nitrogen for 2 h to obtain a 0% DO reference point, as described by Butler et al. [46]. The DO in the sample was measured in 1-second intervals and data were recorded and plotted using the ArrowDO software provided by Lazar Research Laboratories and Microsoft Excel v16.0 (Microsoft, 216 Redmond, WA. USA). Dissolved oxygen curves were compared between species. There were at least 3 biological repetitions for each species, i.e. batches were derived from different cohorts and were tested on different days. Each cohort also included between 2 and 6 technical repetitions with different batches of 200 pupae that were derived from the same cohort.

Irradiation of pupae in hypoxia *versus* normoxia

Pupae of all three species were collected in 4-h windows to ensure uniform pupal age of 40–44 hours for both *Aedes* species, and 20–24 hours for *An. arabiensis*. We chose these age groups as these represent the last hours before they begin to emerge into adults (*An. arabiensis* has a much shorter pupal duration than *Aedes* spp.). *Aedes* pupae were sexed based on pupal size dimorphism using a glass pupal sorter [47] and sex was verified under a stereomicroscope. Pupae of *An. arabiensis* were sexed visually using a stereomicroscope. Males were kept for treatment and females were placed in individual tubes for emergence to ensure virginity for later mating. Male pupae were counted into batches of 80 and were placed either in 0.5 ml Eppendorf tubes in water (for the hypoxic conditions) or into the center (2 cm diameter ring made with hot-melt adhesive) of standard 100 mm × 15 mm Petri dishes (for normoxic (21% O_2) conditions) for irradiation. Higher densities of pupae were used to ensure hypoxic conditions of < 0.5% DO after 30 min in all three species, as was seen in the previous experiment. The Eppendorf tubes were then closed 30 min prior to irradiation treatment to ensure hypoxia (< 0.5% DO) within the tubes. The pupae in both the hypoxic and normoxic treatment groups were irradiated at the same time. At least three technical repetitions and three biological repetitions were performed for all doses in all species.

Radiation treatments were performed in a Gammacell 220 (Nordion Ltd, Kanata, Ontario, Canada), with a current dose-rate of 84 Gy/min. The dose uniformity ratio within the volume used for the irradiators was 1.05 or less. Treatments and controls were performed in either hypoxic or normoxic conditions. The doses used were selected according to the expected dose required to induce 50–100% sterility in each strain: 20, 55, 70, 90 and 100 Gy for *Ae. aegypti*; 20, 35, 55 and 70 Gy for *Ae. albopictus*; 40, 75, 90, 110 and 120 Gy for *An. arabiensis* respectively.

Dosimetry

The dosimetry system used to verify the dose received by the batches was based on Gafchromic HD-V2 and MD-V3 film (Ashland Advanced Materials, Bridgewater NJ, USA) following the protocol of the IAEA [48]. Three films of either HD film (for doses > 50 Gy) or MD film (for doses < 50 Gy) were packed in aluminium envelopes (to avoid getting wet) and placed directly above and below the pupae samples. The temperature near the sample and films was measured before and after radiation exposure. Films were read with an optical density reader after 24 h of development.

Assessment of induced sterility

Following irradiation, 50 males were randomly selected from each treatment group and were placed in a 15 × 15 × 15 cm Bugdorm® cage (MegaView Science Co. Ltd., Taichung 40762, Taiwan) for emergence. Fifty virgin females were added to each cage when the adults reached 2 days of age, and were allowed to mate for 3 days before they were provided with 2 blood meals on consecutive

days (days 6 and 7 post-emergence). Oviposition cups containing water and germination papers (*Aedes* spp.) or filter papers (*Anopheles*) were added to each cage on day 8 for *en masse* egg collection (on days 9 and 10 post-emergence) following routine rearing protocols [44]. Egg papers from *Ae. aegypti* and *Ae. albopictus* were collected, matured (slow-dried over 4 days) and stored for 10 days before hatching. The *An. arabiensis* eggs were collected and hatched the same day. The total number of eggs and the number of first-instar larvae (L1) were counted for each treatment group to derive the hatch rate which was determined by counting the number of hatched and un-hatched eggs using a stereomicroscope. The residual fertility was calculated as a percentage of the control fertility [49]. Induced sterility (IS) was calculated by subtracting the RF from 100%. For overall results for each mosquito strain, all data from the biological and technical repetitions were pooled to attain the mean IS, and the median and upper and lower quartiles shown in the figures.

Statistics

The dynamics of the O_2 water concentration was assessed using a mixed effect binomial model, with the time, mosquito species and their first order interaction as fixed effects and the repeats as a random effect using the *lme4* package in R [50]. The response variable was either the raw O_2 water concentration or its log-transform ($\log(c+1)$). The best model was identified using the corrected Akaike's criterion (AICc) [51].

Binomial linear mixed effect models were used to analyze the impact of hypoxia and irradiation on the hatch rate (HR). The treatment regimen, the radiation dose and their first order interaction were used as fixed effects and the repetitions as random effects. The best model was selected on the basis of the lowest corrected Akaike's information criterion (AICc), and the significance of fixed effects was tested using the likelihood ratio test [52]. All data were analyzed using the R language version 3.2.1 [53].

Results

Measurement of oxygen depletion by pupae in water

Data on oxygen depletion by pupae in water are presented in Fig. 1. The three tested species differed significantly in their response to a closed aqueous environment. *Anopheles arabiensis* depleted the water of DO the fastest with levels plummeting to below 0.1% in under 10 min. The aedine pupae both required longer times to reach equally low DO levels, with *Ae. albopictus* reaching 0.1% DO in between 20–25 min, and *Ae. aegypti* requiring 30–40 min to reach DO levels under 0.3%. All pupae batches (all repetitions and all species) depleted DO levels to under 0.5% within 30 min, and thus 30 min was used to ensure this level of hypoxia for the following irradiation experiments.

The best statistical model to compare the three species responses was the full model with all fixed effects and using the log-transformed O_2 concentration. However, the log-transformation improved the fit much more for *Aedes* species than in *Anopheles*, showing that the trend of DO reduction was much more linear in *An. arabiensis* which is also clear in Fig. 1, while the trend in both aedine species was non-linear. The DO content dropped significantly faster in *An. arabiensis* than *Ae. albopictus* ($P<0.0001$) and again significantly faster in *Ae. albopictus* than *Ae. aegypti* ($P<0.0001$).

Irradiation of pupae in hypoxia *versus* normoxia

Dosimetry

The dosimetry confirmed that all doses received were within the 5% confidence interval of the dosimetry calibration.

Dose-response Ae. aegypti

Mean induced sterility (IS) for this species spanned from 60% at 20 Gy, to 100% at 90 Gy (and 110 Gy) following exposures in normoxic conditions. Mean induced sterility (IS) decreased significantly when irradiated in hypoxic conditions (45% to 99%) at the same doses (Fig. 2).

The best model included all fixed effects and the interaction between oxygen concentration and radiation dose. The hatch rate reduced with increasing dose ($P<0.0001$, Table 1), but hypoxia only had a marginally "protective effect" against irradiation at lower doses, ($P=0.05$). However, this protective effect increased significantly with dose ($P<0.0001$).

Dose-response Ae. albopictus

Mean induced sterility for this species ranged from 74% at 20 Gy, to 99.7% at 70 Gy following irradiation in normoxic conditions, compared to 21% IS and 84% in hypoxic conditions at the same doses.

The best model included all fixed effects and the interaction between oxygen concentration and radiation dose. The hatch rate reduced with increasing dose ($P<0.0001$, Table 2), but hypoxia had a "protective effect" against irradiation ($P<0.0001$). This protective effect increased with dose ($P=0.02$). Hypoxia alone did not change the hatch rate in the control groups ($P=0.77$) (Fig. 3).

Dose-response An. arabiensis

The difference in mean induced sterility for this species when comparing results following irradiation in

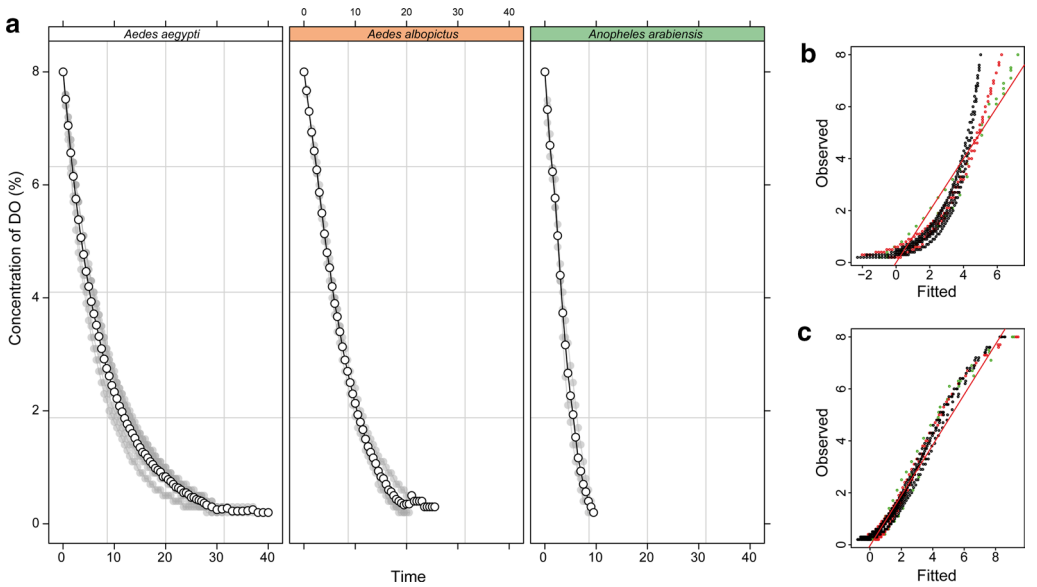

Fig. 1 **a** Dissolved oxygen uptake by *Aedes aegypti*, *Aedes albopictus* and *Anopheles arabiensis* submerged in water. Dissolved oxygen (DO) level in percent (%) over time (minutes). **b** Raw O_2 concentration. **c** Log-transformed O_2 concentration (log(c + 1)). *Key*: black dots, *Ae. aegypti*; red dots, *Ae. albopictus*; green dots, *An. arabiensis*

normoxic versus hypoxic conditions was less pronounced at the lowest doses tested (38% IS at 40 Gy in normoxia *versus* 33% IS at the same dose in hypoxia) but most pronounced of all three species tested at the highest dose of 120 Gy (92% IS in normoxia *versus* 76% IS in hypoxia), (Fig. 4).

The best model included all fixed effects and the interaction between oxygen concentration and radiation dose (Table 3).

The hatch rate reduced with increasing dose ($P < 0.0001$, Table 3). The protective effect of hypoxia against irradiation increased with the dose ($P < 0.0001$) and was even not observed at 40 Gy (leading to an artificial negative effect of hypoxia in the model that cannot be read without accounting for the interaction). Hypoxia alone did not change the hatch rate in the control groups ($P = 0.98$).

Discussion

The oxygen depletion by pupae when submerged in water was clearly demonstrated in our study. It was surprising to see how differently the pupae of the three tested species coped with submergence in water and how quickly the DO was consumed from the surrounding water. However, after reviewing available literature on aquatic organisms and the evolutionary, behavioral and anatomical differences of the three species, the respiratory differences become more evident.

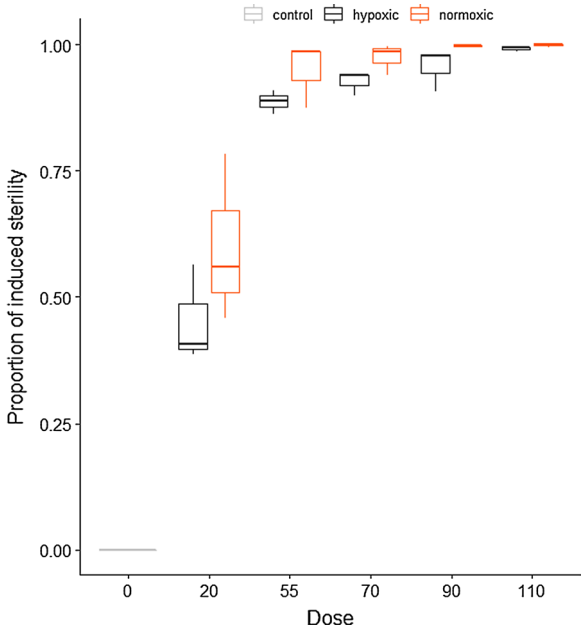

Fig. 2 Dose-response curves for *Aedes aegypti* male pupae following irradiation in normoxic and hypoxic conditions. The box plot shows the median and upper and lower quartiles

In terms of respiratory capabilities, animals can be classified as oxy-regulators and oxy-conformers, depending on their ability to respond to hypoxic conditions [54]. The oxygen consumption rates of oxy-conformers depends

Table 1 Fixed-effects coefficients of a mixed-effect Gaussian model of the impact of radiation dose on the hatch rate in *Aedes aegypti*

Fixed effects	Value	SE	*z*-value	*P*-value
Intercept	0.490346	0.076284	6.43	1.29e−10***
Hypoxia	0.151128	0.078640	1.92	0.0546
Dose	−0.067274	0.001812	−37.12	< 2e−16***
Hypoxia*Dose	0.020379	0.002037	10.00	< 2e−16***

***$P \leq 0.001$

Abbreviation: SE, standard error

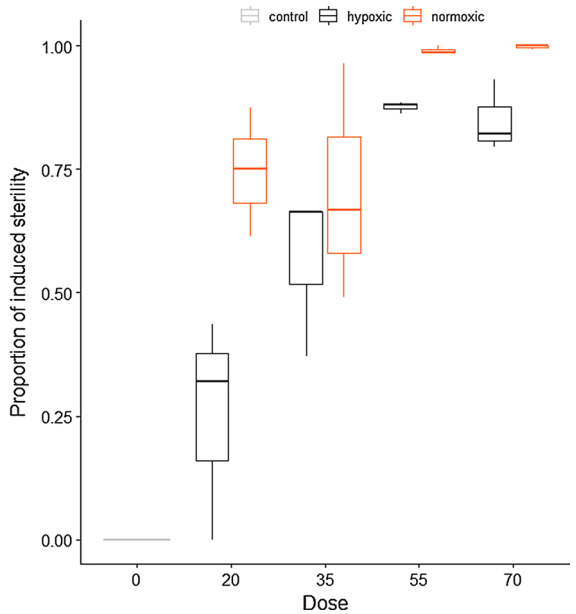

Fig. 3 Dose-response curves for *Aedes albopictus* male pupae following irradiation in normoxic and hypoxic conditions. The box plot shows the median and upper and lower quartiles

Table 2 Fixed-effects coefficients of a mixed-effect binomial model of the impact of radiation dose on the hatch rate in *Aedes albopictus*

Fixed effects	Value	SE	*z*-value	*P*-value
Intercept	−0.290276	0.249733	1.162	0.2451
Hypoxia	0.889215	0.205254	4.332	1.48e−05***
Dose	−0.068176	0.006287	−10.845	< 2e−16***
Hypoxia*Dose	0.014788	0.006650	2.224	0.0262*

*$P \leq 0.05$; ***$P \leq 0.001$

Abbreviation: SE, standard error

on the surrounding oxygen levels, i.e. they demonstrate a respiration rate proportional to the surrounding oxygen tension, whereas in contrast, oxy-regulators maintain

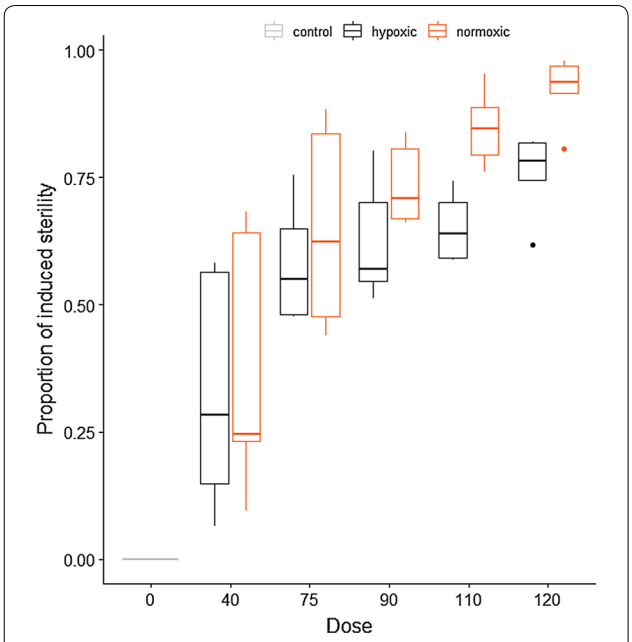

Fig. 4 Dose-response curves for *Anopheles arabiensis* male pupae following irradiation in normoxic and hypoxic conditions. The box plot shows the median and upper and lower quartiles

Table 3 Fixed-effects coefficients of a mixed-effect Gaussian model of the impact of radiation dose on the hatch rate in *Anopheles arabiensis*

Fixed effects	Value	SE	z-value	*P*-value
Intercept	0.825774	0.206693	3.995	6.46e−05***
Hypoxia	−0.476885	0.128439	−3.713	0.000205***
Dose	−0.028555	0.001238	−23.064	< 2e−16***
Hypoxia*Dose	0.013450	0.001578	8.522	< 2e−16***

***$P \leq 0.001$

Abbreviation: SE, standard error

their oxygen consumption rates independently of oxygen levels, until a critical oxygen tension is reached (a threshold level) at which point they can possibly convert into conformers [55]. Considering the results in this study, it is proposed that *An. arabiensis* (and probably all similar species) are oxy-regulators, as their respiration rates maintain constant despite DO levels decreasing around them, producing a linear response, while both *Ae. albopictus* and *Ae. aegypti* appear to be oxy-conformers as they seem to be able to downregulate respiratory activities as DO levels start to decrease in their surrounding environment, which is reflected by the non-linear response.

Our findings are supported by other biological differences described in available literature that are relevant to oxygen usage and respiratory activities for mosquitoes. Difference in these mosquito species are also reflected

in their diving behavior. Mosquito pupae also use gas in their ventral air space (VAS) to be buoyant, and they lose this buoyancy when submerged under water [56]. *Culex pipiens* and *An. stephensi* are, for example, positively buoyant and typically make shallow, and short-duration dives, while many aedine species (for example *Ae. aegypti*, *Ae. albopictus* and *Ae. triseriatus*) tend to make deeper, and longer duration dives [56]. Although diving generally serves to escape predation, the enhanced diving capabilities in some aedine species is suggested to help keep the pupae from being washed out of their breeding container, or other shallow habitat [56].

The environmental history of insects may be an important factor that shapes the response to exposures in hypoxia [57]. The differences in tolerance to low oxygen environments may well have been an evolutionary response for some aedine species and now reflect their preferred small, shallow breeding sites often containing organic pollutants and microbial depletion of DO, whereas this adaptation, historically, was not necessary for the anopheline species which often select cleaner, larger bodies of water for breeding. However, in the recent decades, more and more reports describe the adaptation of *An. arabiensis* to a wider variety of breeding sites, including populations that have adapted to more polluted, urban habitats [58]. The strain of *An. arabiensis* used in this study was originally collected from larger, clean water bodies in Dongola, Norther Sudan before 2010. Preferred larval breeding sites included riverbanks, grassy knolls (flooded soil terraces near to the river), 'khors' (seasonal tributary channels), flood pools and wells [59]. It would be interesting to assess the tolerance to low oxygen environments in *An. arabiensis* populations collected more recently, from urban, shallow, and more polluted larval breeding sites to test the hypothesis that oxy-regulatory capacity evolved based on environmental factors in larval habitats. This physical adaptation and higher tolerance of some *Aedes spp*. to low oxygen environments- and thus their ability to stay submerged for longer periods may be supported by the structure of their tracheal branches which have been found to be different (and significantly larger), for instance, in *Ae. togoi* compared to *An. sinensis* [41].

Understanding the respiratory behavior in mosquito pupae when developing protocols for pupal irradiation, especially in large numbers, and the effects of low oxygen environments on dose-response is important as it is clear that dense pupae samples in water can create a hypoxic environment which provides radio-protection. This means that the dose used to induce the target sterility in small batches of pupae in air, is likely not sufficient for larger, more dense batches of pupae in water, and this could result in the release of sub-sterile males during a SIT programme. In addition to this, it is also important to note that oxygen solubility changes with temperature (DO increases as water temperature decreases), as does the metabolic rate of living organisms (generally metabolism decreases as temperature decreases). Therefore, the rate at which DO becomes depleted may change at lower temperatures. Interestingly, in some aquatic insect species (in the case of *Ilyocoris cimicoides*), only aerial respiration changed with temperature, while aquatic respiration did not [60]. This still needs further evaluation to better understand submerged pupal respiratory behavior in varying temperatures and hypoxia.

Following the assessment of DO depletion by pupae in water in the present study, and the results showing how quickly this occurs, a possible reason for varying sterility results following irradiation of pupae in water *versus* air, and in small batches *versus* large numbers [39, 40], becomes apparent. Thus, a reassessment of the dose-response in pupae was needed for both hypoxic and normoxic environmental conditions during irradiation treatments with the aim of standardizing irradiation protocols for mosquito pupae sterilization.

Most of the DNA damage produced by ionizing radiation comes from free radicals generated during the radiolysis of water, producing DNA strand breaks and other types of lesions that can be cytotoxic or mutagenic. Free radical-induced DNA damage is repaired by an efficient process involving several proteins [61]. These free radicals play an important role once they activate the processes of DNA repair. Therefore, initiating this process prior to radiation exposure could provide an adaptive resistance during irradiation. A study exposing insect cell cultures to X-rays found that, in these cells, the DNA damage and resistance to irradiation (in terms of survival) were greater than that of mammalian cells [62]. The authors' assumption is that the abundance of free amino acids in the haemolymph can play a role in protecting the cell and supporting the repair machinery.

Irradiation in nitrogen (anoxia) has been assessed in *An. gambiae* (pupal stage) and *Culex quinquefasciatus* (pupal and adult stages) and it was reported that this did not have any, or very little, beneficial effects, and that higher doses were required to achieve the target sterility level [35, 36]. Another study showed that, in *Ae. aegypti* (pupae and adults), irradiation at 35, 70 and 100 Gy in nitrogen resulted in males equally competitive as non-irradiated males, compared to males irradiated in air that were not as competitive [63]. However, no known reports exist to date of the irradiation of mosquito pupae in oxygen depleted water and its effects on fertility and adult male biological quality.

Hallinan & Rai [63] demonstrated equal competitiveness in sterile and fertile *Ae. aegypti* males when

adults were irradiated in nitrogen at 70 Gy and 100 Gy as compared to in air. At a lower dose of 35 Gy, the partially sterile (~60–70% sterile) males were three time more competitive than untreated males [63]. However, the samples were irradiated at the same dose in the two atmospheric conditions, meaning that the mosquitoes irradiated in nitrogen may have been more competitive, but are likely to have been less sterile, making these results of little use. Nevertheless, the authors' suggestion that irradiation in nitrogen (anoxia) may be protective and resulting sterile males more competitive are likely to be true, as this was shown by Hooper [64] for *Ceratitis capitata* pupae, *Rodnius prolixus* [65] and *Bactrocera olea* (*Dacus olea*) [66] following irradiation in nitrogen *versus* air, where the irradiation dose was adjusted to achieve equal sterility levels and competitiveness was subsequently compared. Considering the existing information in other insect species, there is a chance that irradiation of mosquito pupae in oxygen depleted water or adults in nitrogen using optimal handling methods can improve sterile male competitiveness in the field. It is therefore, a priority to investigate this topic in further studies.

Among the mosquito species evaluated in this study, the greatest effects induced by hypoxia during irradiation was seen in *An. arabiensis*. This species may generally be more sensitive to oxygen effects during irradiation, or the radioprotective effects may have been highest in this species because the pupae depleted the water of DO the fastest, and thus were kept in lower DO levels for longer prior to irradiation than the two other species tested. It is also important to note that *An. arabiensis* was exposed the longest to radiation, as they require a higher dose for sterilization, which may partially explain why differences in IS were greater between the hypoxia and normoxia treatment groups.

In general, the doses needed for >99% sterility in this study were relatively high compared to results reported elsewhere. Doses of 90, >55 and >120 Gy were needed to achieve over 99% IS in *Ae. aegypti*, *Ae. albopictus* and *An. arabiensis*, respectively, whereas former studies with the same strains have reported lower doses needed for this level of sterility: for *Ae. aegypti* (Brazil strain), between 35 and 50 Gy were needed using X-ray to reach 98–100% sterility (Maylen Gomez Pacheco, pers. comm.); for *Ae. albopictus* (Rimini strain) 35 Gy were sufficient using a cesium-137 source [67], and 40 Gy using X-ray [39, 40]; and for *An. arabiensis* (Dongola strain), 110 Gy were required using the same Gammacell 220 with a cobalt-60 source [23, 67]. One of the possible factors that could lead to these differences could be the oxygen content in the canister environment, or in the water during exposure.

Conclusions

Other than the atmospheric conditions, other factors that may contribute to differences in the reported dose-response in pupae could include exposure temperature (which also effects respiration and DO concentrations in water), differences in dose-rate of the irradiators, differences in pupal age and thus radiosensitivity, changes in the strains' biology over many generations of colonization and inbreeding or different genetic backgrounds among populations [39, 40]. Further research to look deeper into these possible factors is expected to aid in the development of standardized protocols for mosquito irradiation in the frame of the SIT to combat these important vector species.

Abbreviations

AW-IPM: Area-Wide Integrated Pest Management; DO: dissolved oxygen; FAO: Food and Agriculture Organisation; IAEA: International Atomic Energy Agency; IPCL: Insect Pest Control Laboratory; IS: induced sterility; SIT: sterile insect technique; VAS: ventral air space.

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Authors' contributions

HY conceptualized the experimental designs for the experiments and drafted the original manuscript. CC assisted in the development of the tools and methods for the irradiation in hypoxia. AP was responsible for the calibration, verification and assessments of the irradiator and irradiation processes. DOC, MS and AP contributed significantly to the written content and later versions of the manuscript. GW and AA contributed to the design of the DOC measurement experiment and equipment, and provided materials including the calibration liquids and methods. CK, WM, NSBS and TW provided all live material following standardized rearing procedures and assisted in data collection. HY and HM carried out the experiments. JB and HM contributed to the experimental designs and carried out the statistical analyses. JB supervised and supported the project. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study including all dosimetry reports are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1] Insect Pest Control Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna International Centre, P.O. Box 100, 1400 Vienna, Austria. [2] Food and Environmental Protection Laboratory, Joint FAO/IAEA Division of Nuclear Techniques

in Food and Agriculture, International Atomic Energy Agency, Vienna International Centre, P.O. Box 100, 1400 Vienna, Austria. [3] Soil and Water Management & Crop Nutrition Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna International Centre, P.O. Box 100, 1400 Vienna, Austria. [4] Institute for Insect Biotechnology, Department of Insect Biotechnology in Plant Protection, Justus-Liebig-University Gießen, Winchester Str. 2, 35394 Giessen, Germany.

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Radiation dose-rate is a neglected critical parameter in dose–response of insects

Hanano Yamada[1,2]✉, Vanessa S. Dias[1]✉, Andrew G. Parker[1,3], Hamidou Maiga[1], Carina Kraupa[1], Marc J. B. Vreysen[1], Wadaka Mamai[1], Marc F. Schetelig[2], Nanwintoum S. Bimbilé Somda[1] & Jeremy Bouyer[1]

Reproductive sterility is the basis of the sterile insect technique (SIT) and essential for its success in the field. Numerous factors that influence dose–response in insects have been identified. However, historically the radiation dose administered has been considered a constant. Efforts aiming to standardize protocols for mosquito irradiation found that, despite carefully controlling many variable factors, there was still an unknown element responsible for differences in expected sterility levels of insects irradiated with the same dose and handling protocols. Thus, together with previous inconclusive investigations, the question arose whether dose really equals dose in terms of biological response, no matter the rate at which the dose is administered. Interestingly, the dose rate effects studied in human nuclear medicine indicated that dose rate could alter dose–response in mammalian cells. Here, we conducted experiments to better understand the interaction of dose and dose rate to assess the effects in irradiated mosquitoes. Our findings suggest that not only does dose rate alter irradiation-induced effects, but that the interaction is not linear and may change with dose. We speculate that the recombination of reactive oxygen species (ROS) in treatments with moderate to high dose rates might minimize indirect radiation-induced effects in mosquitoes and decrease sterility levels, unless dose along with its direct effects is increased. Together with further studies to identify an optimum match of dose and dose rate, these results could assist in the development of improved methods for the production of high-quality sterile mosquitoes to enhance the efficiency of SIT programs.

For the application of the sterile insect technique SIT[1], insect species targeted for control such as disease-transmitting mosquitoes are mass-reared in a factory. The harmless mosquito males are then separated from blood-sucking females. Subsequently, the male mosquitoes are irradiated with X or gamma radiation to render them reproductively sterile by causing germ-cell chromosome fragmentation that leads to dominant lethal mutations, resulting in imbalanced gametes, the inhibition of mitosis and the ultimate death of the embryo[2]. These sterile males are then transported and released into the area in which the wild populations of the target vector are to be suppressed or eliminated. This strategy, involving sustained sterile male releases, aims to induce sterility in the target insect population and to reduce its density with each generation without affecting the environment and other non-target organisms[1]. For sterile males to succeed in the field, they need to maintain physical quality despite major stressors such as artificial rearing conditions, handling, and radiation exposure. Particularly challenging is the irradiation process where the goal is reliably to achieve near to total sterility whilst keeping the level of off-target, somatic damage as low as possible. Understanding the mosquito's radiation biology and factors that may alter dose–response is crucial to develop standardized irradiation protocols to produce high-quality sterile males. In particular, an acceptable level of consistency in dose–response data is a prerequisite. In this paper, we assess the long neglected topic of dose rate as a variable in insect irradiation, and its implications in the use of nuclear techniques as a method of insect "birth control". Improving irradiation techniques will contribute to the control of disease vector populations that are responsible for spreading potentially fatal illnesses such as malaria, dengue fever, yellow fever, Zika, and many other arboviruses[3,4].

[1]Insect Pest Control Laboratory, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Friedensstrasse 1, 2444 Seibersdorf, Austria. [2]Department for Insect Biotechnology, Justus-Liebig-University Gießen, Winchester Str. 2, 35394 Gießen, Germany. [3]Present address: Roppersbergweg 15, 2381 Laab im Walde, Austria. ✉email: h.yamada@iaea.org; V.Dias-De-Castro@iaea.org

Therefore, the question remains whether dose–response changes with the dose rate at which a target dose is administered? The answer can be different depending on the scientific background of the person asked and depending on the angle from which this topic is observed. An entomologist who uses ionizing radiation to sterilize insects for the SIT is likely to be of the opinion that dose–response remains the same, no matter the dose rate[5]. This would make the total absorbed dose the only factor that matters[6], which is in line with the 'one-hit' ionizing event hypothesis[7]. On the contrary, a radiotherapist in oncology could tell you that the dose does not equal dose in terms of biological effects, and dose rate does have a significant impact on irradiation outcome[8–14]. There are many applications of irradiation, all with different goals and desired outcomes. The big difference in these fields of research is the magnitude of the radiation doses that are being applied. Generally, the more complex an organism, the more radiosensitive it is. A human exposed to doses exceeding 10 Gy (10 Sv) will very likely die[15]. Patients undergoing radiotherapy receive doses fractioned into 1–2 Gy at the target site (e.g., a tumor) per treatment, depending on the tissue treated. Insects, however, are very diverse and can tolerate doses of up to 600 Gy, such as some moth (Lepidoptera) species that not only survive such doses but remain partially fertile[16].

During the last decades, numerous irradiation studies in mosquitoes including some recent research on mosquito SIT have reported widely divergent dose–response results for the same species[17]. Several factors that affect dose–response in mosquito species currently targeted by the SIT (namely *Anopheles arabiensis, Aedes aegypti,* and *Ae. albopictus*) have been investigated at the Insect Pest Control Laboratory IPCL of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, such as handling methods, life stage, pupal age, strain origin, ambient temperature, and atmosphere during irradiation[18,19]. However, additional factors that affect dose response are suspected and require further scrutiny. Dose rate effects have been investigated only sporadically in historic publications, and the topic has mostly been neglected in area-wide integrated pest management programmes that include an SIT component.

Importance of dose rate. Some studies are available, investigating dose rate effects on lethality[20–22], insect quality[5,23–25], and sterility[21,25–28] but the results have largely been contradictory.

There are on the one hand a series of studies that showed higher damage with higher dose rates when applying a high total absorbed dose. Gonen and Calderon[28] found that dose rates of 110 Gy min^{-1} administered to males of the cacao moth *Ephestia cautella* induced greater sterility than 28 Gy min^{-1} with total absorbed doses of 200, 300, and 400 Gy. A similar trend was observed in the sawtoothed grain beetle *Oryzaephilus surinamensis*[21]. A study by Haverty and Ware[23] indicated that mortality of the pink bollworm *Pectinophora gossypiella* increased as the dose rate increased. Jeffries and Banham[23] demonstrated that increasing dose rate administered to the sawtoothed grain beetle, the confused flour beetle *Tribolium confusum*, and the wheat weevil *Sitophilus granarius* increased detrimental biological effects. Other dose rate dependent negative effects were shown in all life stages of the bruchid beetle *Callosobruchus maculatus*[29], in food storage pests[21] and in the codling moth *Cydia pomonella*[20,30].

Other studies, however, did not corroborate the above results. Ernawan et al.[25] assessed a series of dose rates in *Aedes aegypti*, but besides affecting some quality parameters such as longevity and mating competitiveness, there was no difference in induced sterility. La Chance[7] argued that the induced sterility at a given dose should be the same no matter the dose rate, and Hooper[27,31] found no relationship between dose rate and induced sterility, nor insect quality in two fruit fly species. Subsequently, Collins et al.[5], tested a dose of 70–75 Gy at increasing dose rates of 5, 7, 26, 57, and 80 Gy min^{-1} on the sterility in the Queensland fruit fly *Bactrocera tryoni* to settle the question of whether dose rate affects dose–response and found no effects on sterility or insect quality. Since the Collins et al. study, the topic has been placed on the backburner and very little has been reported since.

There is, to date, compelling data for and against the one-hit ionizing event hypothesis from an entomological standpoint. This does not imply that one or the other studies may be flawed. However, the studies may have only uncovered pieces of the whole dose rate/dose–response picture. Other factors may influence the degree of dose rate effects. Although hypotheses surround the mechanisms, there has been no research questioning a possible relationship between dose, dose rate, and their combined effects on dose–response. In this study, an attempt was made to control all or most variable factors, including dose rate and dose using attenuators and multiple irradiators (of the same make and model), to investigate the effects of dose rate and define the relationship, if any, between dose, dose rate and induced sterlity, and to uncover the missing pieces to the question "does dose equal dose".

Materials and methods

Biological material. The *Ae. aegypti* strain originated from field collections in Juazeiro, (Bahia), Brazil and was transferred to the IPCL from the insectary of Biofabrica Moscamed, Juazeiro, Brazil in 2016. The *Aedes* strains have been maintained following the "Guidelines for Routine Colony Maintenance of *Aedes* mosquitoes"[32].

The Dongola strain of *An. arabiensis*, originating from Dongola, Northern State, Sudan, was donated by the Tropical Medical Research Institute, Khartoum, Sudan in 2010 and maintained at the IPCL following the Anopheline mass rearing guidelines[33].

Radiation sources and dosimetry. Three Nordion Gammacell 220 (GC 220) irradiators were used with initial dose rates of 1.1, 4.3, and 84 Gy min^{-1}. In addition, the dose rate of the 84 Gy min^{-1} irradiator was attenuated with a set of attenuators to 8.3, 26.3, and 38.3 Gy min^{-1}. During the experiments the dose rates of each source decayed and the values were noted in each experiment.

Calculation of dose rates and dosimetry. Dose rates for each of the three GC220s were assessed and verified using a Farmer type 0.18 cm^3 free air ionization chamber (10X6-0.18, RadCal Corporation, Monrovia, CA. USA) in conjunction with a digitizer and electrometer (AccuDose Model 9660A) as a reference dosimetry system to

measure the dose rate and accumulated dose at a designated reference position. The ion chamber system was calibrated by the John Perry Laboratory (St George's University Hospital Trust, London) with traceability to the National Physical Laboratory with a calibration factor of 1.0 and uncertainty of 3.3% (k = 2) in the energy range 40–1250 keV.

The irradiation field in the GC220 irradiation chamber has previously been mapped[34]. The samples in this study were placed in the center of the chamber, and in the center of a petri dish within a 2 cm diameter ring. The variation of dose rate within the sample was less than 1%.

The dosimetry system used to verify the dose received by the batches was based on Gafchromic HD-V2 and MD-V3 film (Ashland Advanced Materials, Bridgewater NJ, USA) following the protocol of IAEA[34]. Three films of either HD film (for doses > 50 Gy) or MD film (for < 50 Gy) were packed in aluminium envelopes to protect them from moisture (Aluminum Laminate Detector Pouch FWT-81, Far West Technologies Inc., Goleta, CA, USA) and placed directly above and below the pupae samples. The temperature near the sample and films was measured before and after radiation exposure. Films were read with an optical density reader (DoseReader4, RadGen Ltd, Budapest, Hungary) after 24 h of development.

Experiment 1: high dose rate vs. low dose rate (2 species, 2 doses, 2 dose rates). *Sample preparation and irradiation.* Pupae of *Ae. aegypti* (aged 44–48 h) and *An. arabiensis* (aged 20–24 h) were collected and were batched into 3 repetitions of 30 individuals per species, per treatment. Radiation treatments were performed in normoxia, in Petri dishes surrounded by a 4 mm PMMA tube, in either a high activity Gammacell 220 with a dose rate of 84 Gy min^{-1}, or in a low activity Gammacell 220 with a dose rate of 1.005 Gy min^{-1}. The pupae were contained within a 2 cm diameter ring in the center of the petri dish. Two doses were selected according to the expected dose needed for intermediate sterility and high sterility (> 98%): for *Ae. aegypti* these were 40 Gy and 110 Gy, and for *An. arabiensis*, 90 Gy and 130 Gy. Control pupae were handled identically but were not irradiated. Two biological repetitions each with 3 technical repetitions were performed.

Assessment of induced sterility. Following irradiation, males were placed in 15 × 15 × 15 cm Bugdorm cages (MegaView Science Co. Ltd., Taichung 40762, Taiwan) for emergence. 30 virgin females (of the same age) were added to each cage and were allowed to mate for 2 nights, before being bloodfed with fresh porcine blood (*Aedes*) or defrosted bovine blood (*Anopheles*) on 2 consecutive days and were then allowed to oviposit. Egg papers from *Ae. aegypti* were collected, matured (slow-dried over 4 days) and stored for 10 days before hatching. The *An. arabiensis* eggs were collected and hatched the same day. The total number of eggs, (hatched- and un-hatched eggs) were counted for each treatment group using a stereomicroscope to derive the hatch rate. Non hatched eggs were dissected to ensure fertility status. The residual fertility (RF) was calculated as a percentage of the control fertility of each treatment group ($RF = HR_{tx}/HR_c \times 100$). Induced sterility (IS) was calculated by subtracting the RF from 100%.

Experiment 2: dose rate response curve (1 species, 1 dose, 6 dose rates). *Sample preparation and irradiation.* Pupae aged 44–48 h of *Ae. aegypti* were irradiated as described in the previous section "Experiment 1". All samples were irradiated simultaneously; shorter exposures were performed in the high dose rate GC220 during the longer ones in the low dose rate GC220s. Two biological repetitions each with 3 technical repetitions were performed. The following dose rates were used to irradiate all samples with 20 Gy: 1.1, 4.3, 8.3 26.3, 38.3, and 84 Gy min^{-1}. Induced sterility was assessed as described in the previous section "Experiment 1".

Experiment 3: Interaction between dose and dose rate (4 doses × 5 dose rates). *Sample preparation and irradiation.* Pupae aged 44–48 h of *Ae. aegypti* were irradiated as described in the previous section "Experiment 1". All samples (each with 30 pupae) were irradiated within a given time span; shorter exposures were performed in the high dose rate GC220 during the longer ones in the two low dose rate GC220 irradiators. Three biological repetitions, each with 3 technical repetitions, were performed. The following doses and dose rates were used for the irradiation of samples: 10, 20, 40, 70 Gy and 0.4, 1, 7.8, 24.5, 79 Gy min^{-1}, respectively. These dose rates were used due to the availability of gamma sources, and 50%, 70% and 90% attenuators. Induced sterility was assessed as described in the previous section "Experiment 1".

Proof of principle: survey on sterilization doses used in SIT projects against *Aedes* spp. To identify possible reasons for the differences reported in the dose needed to achieve sterility in mosquitoes, a survey was conducted in several mosquito SIT research groups in several countries to see what variable factors may account for these differences. The survey included information regarding the biological factors, handling protocols, irradiator type used, characteristics such as the radiation source, and dose rate.

Statistics. All analyses were performed in R language version 3.2.1[35]. High vs. low dose rate data: Generalized linear mixed models (*glmer* function in lme4 package) were used with radiation dose rate and dose considered as fixed factors and cage (replicates) as a random factor. The full models were checked for overdispersion (using Bolker's function) and for normality and homogeneity of variances on the residuals. The models were simplified using the stepwise removal of terms, followed by likelihood ratio tests (LRTs). Term removals that significantly reduced explanatory power ($p < 0.05$) were retained in the minimal adequate model. The significant interactions were analyzed using the *emmeans* function (in package emmeans).

Dose rate response curve: egg hatch rate data were corrected to induced sterility related to the mean control natural sterility and compared between treatments using analysis of variance (ANOVA) and Tukey's post hoc tests.

A Generalized linear mixed model fit by maximum likelihood was used to analyze the hatch rate data (response variable), using the log of the dose and the hatch rate as fixed effects, and the repeats and technical replicates as random factors[36,37]. Dose rate was used either as a numeric factor or grouped in clusters of similar effect on the hatch rate to account for the non-linear relationship between hatch rate and dose rate. Two groupings were tested, either [0.4; 1; 7.8 Gy min^{-1} or more] or [0.4–1; 7.8; 24.5 Gy min^{-1} or more]. The best model was selected based on the lowest corrected Akaike information criterion (AICc). The likelihood ratio test was used to ascertain the significance of the fixed effects. The R^2 (coefficient of determination) between the observed and predicted values was used to describe the proportion of variance explained by the selected model[38].

Student *t*-test was used to compare lowest and highest dose rate effects (DRE) by dose after fertility data was checked for normality and was log transformed.

Results and discussion

Experiment 1: low dose rate achieved greater sterility than high dose rate at high doses. Three self-contained Co^{60} gamma-ray irradiators of the same make (Gammacell 220, Nordion Inc., Canada, hereafter called GC220) were used for this experiment with dose rates of 84.07 Gy min^{-1}, 4.02 Gy min^{-1} and 1.03 Gy min^{-1}. First, we assessed the effects of high dose rate (84 Gy min^{-1}) versus low dose rate (1 Gy min^{-1}) at two nominal doses (40 Gy and 110 Gy for the mosquito *Ae. aegypti* and 90 Gy and 130 Gy for the mosquito *An. arabiensis*). Irradiated males were mated to untreated females to measure induced sterility in the eggs laid by these females (see M&M for details).

For both species, the low dose rate yielded higher levels of sterility (Supplementary Table S1). In *Ae. aegypti*, a dose of 40 Gy at the high dose rate induced 88.9% sterility, compared to 93.4% following exposure at the low dose rate ($P = 0.02$). A dose of 110 Gy induced near complete sterility at both dose rates, with only 1 and 2 larvae hatching in one of the 6 repetitions from low dose rate and high dose rate treatments, respectively, and thus could not be compared in a meaningful manner. In *An. arabiensis*, 90 Gy induced 69.6% sterility at the high dose rate, and 87.6% at the low dose rate ($P = 0.002$). A dose of 130 Gy at the low dose rate achieved complete sterility, while the same dose at the high dose rate only reached 77.0% of induced sterility (IS) ($P < 0.05$). The differences in the IS following the two treatments was more pronounced in *An. arabiensis*.

The results of this simple experiment were clear, though contradictory to previous reports[20,21,23,28–30]. The low dose rate had a higher impact on the reproductive biology in both species, with higher sterility levels achieved for both medium and high doses (targeting sterility levels of 75% and near 100%). These results were contrary to what was expected based on previously published reports in the literature (cited above), i.e. dose rate effects in insects were generally found to be enhanced as dose rate increases, or the study of Ernawan et al.[25], that showed no dose rate effects on the sterility of *Ae. aegypti* with a total absorbed dose of 70 Gy. However, they were in line with the reports on "inverse dose rate effects", the earliest documented in 1979, in HeLa cells by Mitchell et al.[39].

Experiment 2: at a low dose, increasing dose rates increased sterility. As the two doses tested for *Ae. aegypti* induced a very high level of sterility, making it difficult to measure meaningful differences, a lower dose of 20 Gy was selected that was expected to induce around 50% sterility, and tested against a larger range of dose rates. *Ae. aegypti* pupae were used for this experiment and were irradiated in the three available GC220s at dose rates of 1.1 Gy min^{-1}, 4.3 Gy min^{-1}, and 84 Gy min^{-1} and using 50%, 70%, and 90% dose attenuators (actual attenuation values confirmed as 54.4%, 68.7% and 90.1%), in the high dose rate irradiator, to obtain the following 5 dose rates: 1.1, 4.3, 8.3, 26.3, 38.3, and 84 Gy min^{-1}. Results from this assessment showed the opposite of the previous experiment, with irradiation effects on fertility increasing as dose rate increased (Fig. 1), contradicting our previous results, but supporting those of historic reports described above.

The conclusion that radiation effects on the insect cells increase as dose rate increases is consistent with several studies in insects[20–24,28,30,40]. Damage repair mechanisms may be the underlying factor for direct dose rate effect occurrence[41–43]. The increased sterility caused by increasing dose rates could be a side effect of more indirect damage through reactive oxygen species (ROS), and/or due to the higher number of chromosome breaks per exposure, resulting in more broken chromosome ends, which is thought to have an important role in species with holokinetic chromosomes[7]. It is possible to estimate radiation-induced DNA damage using the comet assay, and it has been shown by Shetty et al.[44] in *Ae. aegypti* that increasing radiation doses result in increased percentage of comet tail DNA. It is questionable whether this assay could pick up small differences in DNA breakage patterns after irradiation at the same dose, but at varying dose rates, but this may be done in future studies.

Experiment 3: dose rate effects are dose-dependent. Finally, a more in-depth study was carried out to assess potential relationships between dose rate and dose using a series of doses (10, 20, 40, and 70 Gy) administered at a series of dose rates (1.1, 4.3, 8.3 26.3, 38.3, and 84 Gy min^{-1}), again using the three GC220s and attenuators.

The results obtained are summarized in Fig. 2a,b and confirm the results of both preliminary studies. At low doses, radiation effects (observed as a reduction in fertility) increased as dose rate increased. In contrast, at higher doses, these effects (fertility) decreased as dose rates increased, with a turning point where dose rate effects reach a plateau before possibly switching to inverse dose rate effects occurring at doses above 30 Gy (Fig. 2a,b). Absolute differences in fertility levels are also clearly seen when expressed as normalized equivalent deviates, NED) at the various dose rates relative to 1 Gy min^{-1} (Fig. 2b). This result also explains how all historic reports on dose rate effects in insects could be coherent.

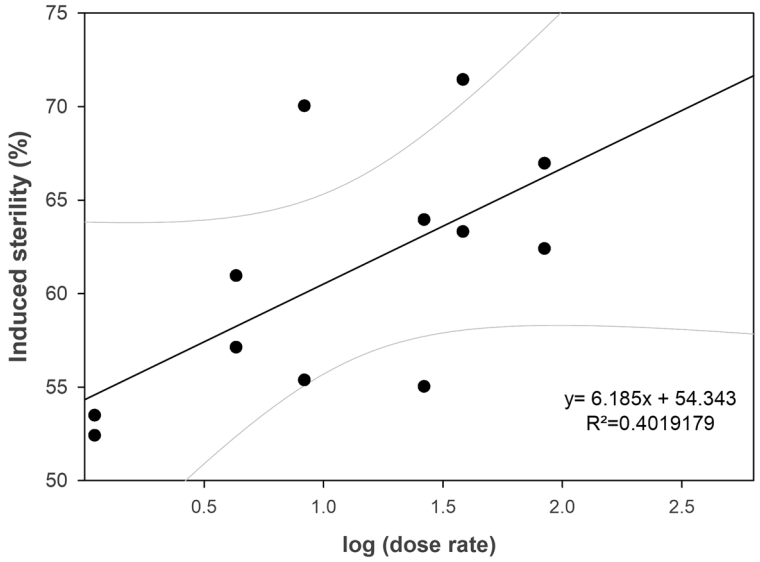

Figure 1. Dose rate response plot showing dose rate effects of *Ae. aegypti* pupae irradiated with 20 Gy on induced sterility in untreated mated females ($P < 0.02$).

As the interaction of dose and dose rate is non-linear, we attempted to depict the relationship by identifying the points where the lowest and highest dose rate effect (DRE) (shown as residual fertility) per dose administered were observed. The results are shown in Supplementary Table S2.

Generally, at lower doses (e.g. 10 and 20 Gy), the DRE increased (sterility increased) as dose rate increased (Fig. 2a). At higher doses (ex. 40 and 70 Gy), DRE decreased (sterility decreased) as dose rate increased. In other words, when identifying which dose rates resulted in the most and least effects on fertility with each dose, these switched when dose increased.

Proof of principle: survey on sterilization doses used in SIT projects against *Aedes* spp. A survey was sent to collaborators and colleagues involved in mosquito irradiation and information requested on irradiation materials and methods. The data indicate that the most obvious difference between SIT projects was the irradiation devices used, and their dose rates and energies. Figure 3 summarizes this information and the dose needed to achieve > 99% sterility in *Aedes* mosquito males (regardless of handling methods and other variable factors that may be present). All data were pooled for *Aedes* species (*Ae. aegypti* and *Ae. albopictus* although *Ae. aegypti* is slightly more radioresistant than *Ae. albopictus*), and for source type (Co^{60}, Cs^{137}, and X-ray) and the doses required for ~ 99% induced sterility were plotted against the dose rate (Fig. 3). Higher doses were required to achieve the same levels of sterility when dose rates increased, except at the very low dose rate of 0.3 Gy min^{-1}. Although other external factors such as handling methods, container materials, pupal age etc. may have also contributed to the differences shown, the data provides a conceptual notion that dose rates play a role in mosquito sterilization.

This rough U-shaped graph is a familiar picture and has been observed in some older publications. The viability in the eggs of the rust-red flour beetle (*T. castaneum*) reduced as dose rate increased, but then viability increased again at very high dose rates[40], giving a V-shaped graph. The authors suggest that the factors that contribute to an increased viability in the embryos at low dose rates (i.e. repair taking place in the cells) are different than those that increase viability at high dose rates (possibly hypoxia induced by high dose rates providing some radioprotection)[40]. Using a hamster model, Vilenchik and Knudson[45] reported that mutagenic effects in somatic and germ cells are generally reduced as dose rate is reduced, showing a direct dose rate effect, but some cell lines show an inverse dose rate effect at very low dose rates, resulting in a parabolic relationship between dependence of induced mutations and dose rate. In the codling moth (*Cydia pomonella*), dose rate effects were assessed at fixed doses, on larval mortality, pupal mortality, and adult emergence[30]. For all three parameters, the relationship between dose rate and biological effects were not linear, with a positive correlation reaching a limit and then switching to a negative correlation, or vice versa, resulting in U-shaped, or inverted U-shaped graphs. Ernawan et al.[25] also observed an increase in longevity as well as mating competitiveness as dose rate increased up to a certain threshold, but then these quality parameters decreased again as dose rates were increased further. Although some of these reports insinuated that dose rate had effects partially dependent on dose, the nature of the relationship was not investigated. What is happening in the observed U-effect? We propose that direct and indirect dose effects are affected by dose rate, i.e. inverse dose rate effects.

Reactive oxygen species (ROS) can be generated as byproducts of the normal metabolism of oxygen and in response to environmental stress, among other factors. When organisms are exposed to ionizing radiation, ROS can be dramatically generated within cells, primarily through the radiolysis of water, which can cause oxidative stress due to insufficient antioxidative protection, and hence significant damage to DNA and other macromolecules. In our simplistic model (Fig. 4), assuming that radiation dose remains the same, we propose that the

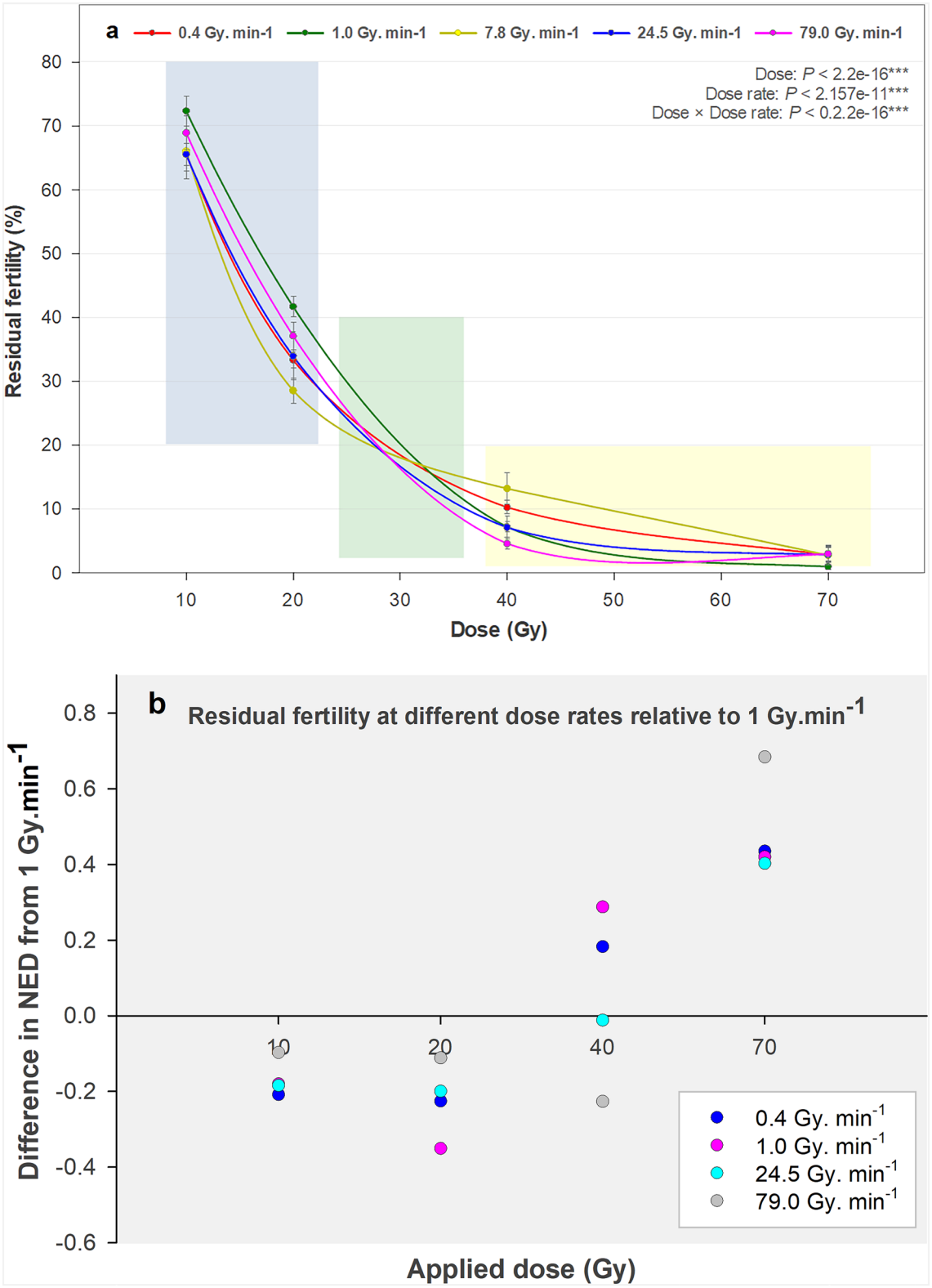

Figure 2. (**a**) Interaction of dose and dose rate: a zone with a positive correlation of the two factors (yellow zone: low doses and increasing dose rates), a negative correlation with inverse dose rate effect (green zone: high doses, with increasing dose rates) and a zone where there are no effects of dose rate (blue zone: mid-range doses). The best model predicting the hatch rate used the log of the dose, the dose rate use as a factor in three groups [0.4; 1; 7.8 Gy min^{-1} or more] and their interaction. It demonstrated that at a low dose, a dose rate of 0.4 Gy min^{-1} or 7.8 or more Gy min^{-1} was more efficient at reducing hatch rate than a dose rate of 1 Gy min^{-1} ($p < 10^{-3}$). The opposite was observed when the dose increased, with a changeover point between 20 and 40 Gy, the dose of 1 Gy min^{-1} becoming the most efficient at inducing sterility ($p < 10^{-3}$). (**b**) Absolute differences in fertility levels (expressed as normalized equivalent deviates, NED) at the various dose rates relative to 1 Gy min^{-1}.

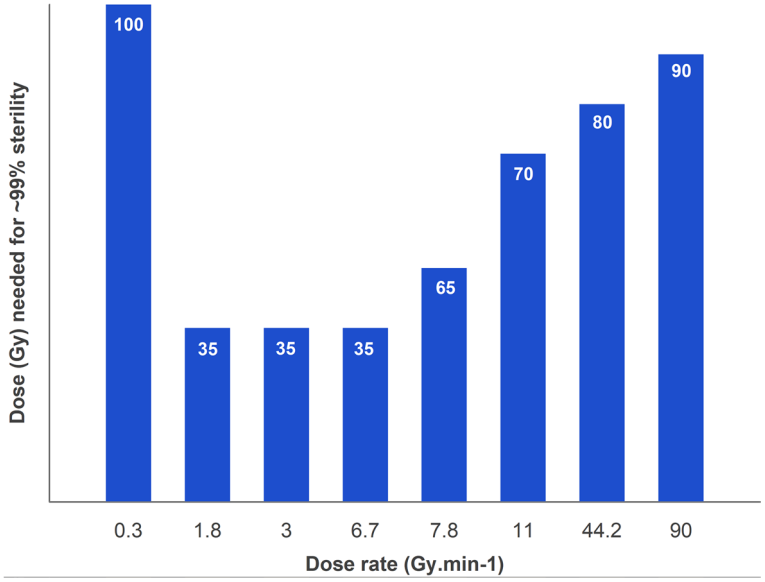

Figure 3. Results from the intercountry survey. Dose required for > 99% induced sterility at different dose rates, regardless of all other parameters and source etc. *Ae aegypti* and *Ae albopictus* data pooled.

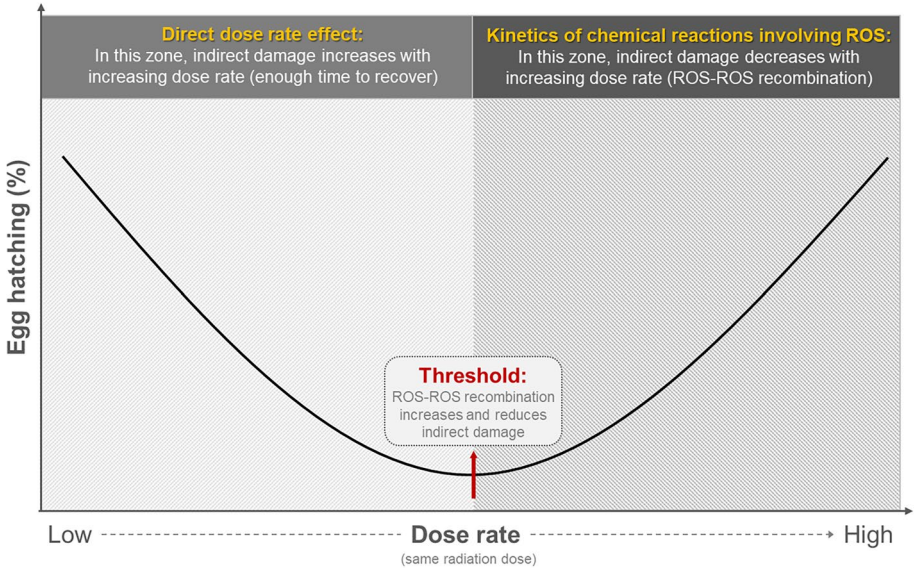

Figure 4. Illustration of the hypothesis for an indirect radiation effects influenced by dose rate, and the differential damage/repair mechanism.

higher the dose rate after the threshold, the more ROS–ROS recombination may be observed. The threshold in our current results, in this particular insect was between 20 and 40 Gy, based on Fig. 2a. After this threshold, radiation damage is attenuated by ROS–ROS recombination but can increase if direct damage (by increasing radiation dose) is increased.We base our explanation of the observed inverse dose rate effects on information found in various oncology references whereby we propose that the indirect effect of irradiation is driven by ROS production, which was reduced due to ROS recombination at higher doses and at high dose rates. Another argument, borrowed from the research on FLASH-RT (ultra-high dose rate radiotherapy)[46], is that irradiation with high dose rates reduces ROS production due to a local transient hypoxia[10]. Under those circumstances (high amounts of ROS being generated in a short time interval with the high dose rate), a high level of sterility could only be achieved by direct effects of irradiation. The increase in sterility could only be achieved by increasing the dose of radiation, which led to more double-stranded breaks through direct effects of radiation[10].

Additionally, the differential dose response could be due to primary and secondary dose rate effects. Studies from before 1958 on dose rate effects (mainly with *Drosophila* sperm) showed that the accumulation of mutations caused by ionizing radiation was independent of dose rate. This general view was altered post 1958 when

Russel[9], and later Phillips[47], found fewer mutations in mouse spermatogonia when radiation doses were given at low dose rates, as compared to high dose rates[43]. At this stage, the assumption was that either there was a primary effect of dose rate on the mutation process or secondary effects such as differential killing of cells depending on their intrinsic sensitivity to mutation induction[43]. The theory of a secondary effect was then countered by the investigations by Russel in mice[9], however the possibilities are so numerous that secondary dose rate effects, although unlikely, cannot be completely dismissed[43].

The two main theories to explain a primary dose rate effect on the mutation process itself are that (1) mutations are a multi-hit phenomena (likely two-break chromosomal aberrations) and, therefore, need multiple radiation events within a limited time, and (2) that mutations are a one-hit event, but the dose rate dictates the probability of repair of permutational damage within the cells[43]. Most reviews are in favor of the second of the two theories.

The two main mechanisms by which dose rate could affect the repair mechanism are that either there is greater damage to the repair mechanisms when exposed at high dose rates rather than low dose rates, or there is a saturation of the repair mechanism capacity, and there is limited time for it to act when the dose rate is high[43].

In mice spermatogonia, it was found that the induced mutation rate approaches a lower limit (ca 1/3 of the maximum value) as the dose rate is reduced from 90 to 0.8 rad min^{-1}, before it reaches a plateau and stays constant as the dose rate is lowered further to 0.001 rad min^{-1}[43,48,49]. In our study, we may also see such a plateau, in which no dose rate effect is seen before the effects are inversed as the dose rate increases or decreases.

In insects, dose rate effects have been investigated in the silkworm[50,51] where two differing effects were described: in early larval stages, high dose rates produced more mutations than low dose rates (type 1 dose rate effect), whereas in later larval stages, the opposite was observed (type 2 dose rate effect). It was suggested that the type 1 effect can be explained by some influence on the repair mechanism (as suggested for the mouse), and type 2 effects result from selective killing[50] or from a more persistent inhibition of repair by lower dose rates[51].

An inverse dose rate effect of ionizing radiation has been reported in human cell response studies[52,53] and it has been observed in some instances (at higher doses) but not in others (i.e. no dose rate effects in lower doses) within the same study with heterogeneous cell populations[54]. One hypothesis explains this effect on the basis of a "window of sensitivity" in the cell cycle[54,55], in which the effects of irradiation is enhanced as the exposure time is increased (hence the dose rate is decreased), whereby an enhancement requires "some kind of saturation" where extra hits to a cell result in less than a proportionate increase in the probability of a damage end point[55]. This type of saturation may result in an inverse dose rate effect via a higher incidence of wasted hits during high dose rate exposures, compared to a lower dose rate[55].

Although it is not fully clear what mechanisms drive the dose rate effects in mosquito dose responses in terms of inducing sterility, there are clues that suggest the existence of a dose rate "region of minimal mutability"[45] also in mosquito irradiation, suggesting that there may be a possibility to pair dose rate and dose, to optimize the response in a way that high levels of sterility and minimal off-target damage can be achieved, producing a better quality sterile male.

Conclusions

Our findings may now go some way to explain why some SIT researchers require much higher doses than others to achieve full sterility in the same insect species. Whereas the observed U-shape when pairing dose and dose rate for radiation sterilization has to be accepted as a reality, the mechanisms behind the dose rate dependent effects in inducing sterility in insects remain speculative but very intriguing. The observed take-home message, i.e. there is undoubtedly a dose rate effect when sterilizing mosquitoes and that this effect is dependent on dose, has also been noted with other insects, such as Lepidoptera. As recently observed in the false codling moth *Thaumatotibia leucotreta* programme in South Africa, declining dose rates of a gamma irradiator resulted in lower sterility levels for a total absorbed dose of 150 Gy (Nevil Boersma, personal communication). This epitomizes the absolute need in operational SIT programmes to implement routine and periodic quality control in terms of biological dosimetry when the source of the irradiators in SIT programmes decays over time, or irradiators are reloaded, the configuration of the irradiation canister is changed in any way that would alter the dose rate, or the irradiator itself is exchanged, as the target induced sterility may not be achieved as expected. Failing to implement these quality control measures might result in the release of male insects that are only sub-sterile, which might prolong the programme unnecessarily or in the worst case, even result in programme failure. However, further research is necessary to better understand the dose dependent dose rate effects in insects.

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Author contributions

H.Y. conceptualized the experimental designs for the experiments and drafted the original manuscript. V.D. contributed significantly to the hypotheses on biological response mechanisms and written content of the original draft and later versions of the manuscript. A.P. was responsible for the calibration, verification and assessments of the irradiator and irradiation processes, and contributed to data analyses. C.K. and N.S. provided all live material following standardized rearing procedures and assisted in data collection. H.Y., H.M. and W.M. carried out the experiments. J.B., M.V., M.S. and H.M. contributed to the experimental designs, contributed to data analysis, carried out the statistical analyses, and contributed to the formulation of hypotheses to explain all results, and contributed to the later versions of the manuscript. J.B. supervised and supported the project. All authors read and approved the final manuscript.

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Correspondence and requests for materials should be addressed to H.Y. or V.S.D.

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Effects of Chilling and Anoxia on the Irradiation Dose-Response in Adult *Aedes* Mosquitoes

H. Yamada[1,2], H. Maiga[1], C. Kraupa[1], W. Mamai[1], N. S. Bimbilé Somda[1], A. Abrahim[3], T. Wallner[1] and J. Bouyer[1]*

[1]Insect Pest Control Laboratory, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna, Austria, [2]Department for Insect Biotechnology, Justus-Liebig-University Gießen, Gießen, Germany, [3]Food and Environmental Protection Laboratory, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna, Austria

The success of the sterile insect technique (SIT) relies on the achievement of high levels of sterility and mating success of the factory-reared sterile males and thus their biological quality, which can be enhanced by the reduction of stress factors encountered during rearing, handling, and irradiation procedures. The achievement of consistent sterility levels requires reliable and standard irradiation protocols. Additionally, mosquito adults require immobilization prior to, and during irradiation to increase processing efficiency and to avoid physical damage caused by movement in restricted space. Common methods for immobilization include chilling and anesthetics such as nitrogen. Here we assessed the effects of chilling and exposure to nitrogen on the irradiation dose-response of *Aedes* mosquitoes, and their downstream effects on some male quality parameters including longevity and flight ability. We found that chilling does not incur damage in the insects in terms of longevity and flight ability when chilling duration and temperature are carefully controlled, and a recovery phase is provided. Irradiation in nitrogen shows high radioprotective effects during irradiation, resulting in reduced induction of sterility. Overall, longevity of males can be improved by irradiating in anoxia, however the exposure to nitrogen itself comes with negative impacts on flight ability. The results reported here will assist in the standardization and optimization of irradiation protocols for the SIT to control mosquito populations of medical relevance.

Keywords: induced sterility, mosquito, irradiation, *Aedes aegypti*, *Aedes albopictus*

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****Correspondence:***
H. Yamada
h.yamada@iaea.org

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1 INTRODUCTION

The sterile insect technique (SIT) (Dyck et al., 2021) is a biological insect population control tactic that reduces the dependence of insecticides and thus agrees with present day concerns regarding human health and the environment. The SIT concept has been in existance since the 1930's, and has been implemented against various crop pests with huge success since the 1950's.

Abbreviations: AW-IPM, Area-Wide Integrated Pest Management; DO, dissolved oxygen; FAO, Food and Agriculture Organisation; IAEA, International Atomic Energy Agency; IPCL, Insect Pest Control Laboratory; IS, induced sterility; SIT, sterile insect technique; VAS, ventral air space.

It was first implemented against mosquitoes in the 1960's with varying results (Dame et al., 2009), however, the technique has more recently regained interest in the fight against malaria, and in response to the Zika virus outbreaks in 2015. Following increasing demands from Member States, the Insect Pest Control Laboratory of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture has been developing the SIT package for select disease transmitting mosquito species, in particular *Aedes aegypti* and *Ae. albopictus* (the main vectors of dengue, Zika, chikungunya among other arboviruses) and *Anopheles arabiensis*, an important vector of malaria. Great progress has been made for each component of the SIT, resulting in the development of equipment, methods and guidelines for mass rearing, sex separation, irradiation, packing, transportation, quality control, release methods, and field trials. The most notable achievements of the past decade are reviewed in (Vreysen et al., 2021).

The success of the SIT relies on the reliable induction of sterility in the target insect population by releasing mass produced sterile males into the field, where they must outcompete wild counterparts to secure mating which results in no offspring. For this, dependable irradiation protocols are required to ensure constant, and high levels of induced sterility, whilst maintaining the highest possible quality in the sterile insects. The irradiation of medically important mosquito species in the frame of the SIT requires the males to be near to fully sterile both to avoid a risk of replacement of the target population (WHO and IAEA, 2020), and to ensure a maximal efficiency of the sterile males given the high reproduction rate of these species (Aronna and Dumont, 2020). This can be achieved by exposing pupae or adults to ionizing radiation- usually in gamma-ray irradiators (Helinski et al., 2009 and references within), and more recently, in X-ray irradiators (Mastrangelo et al., 2010; Yamada et al., 2014; Du et al., 2019; Zheng et al., 2019), and possibly with industrial accelerators producing electron beams (Balestrino et al., 2016), although these devices are currently used mainly in phytosanitary applications (Smittle et al., 1991; Dohino et al., 1997; Todoriki et al., 2006; Koo et al., 2012).

Although there is a high degree of reliability when achieving expected sterility levels by exposure to a known dose according to dose-response studies, some physical factors influence the dose response in mosquitoes, and biological factors also affect their general sensitivity to radiation. Some of these factors have been studied more frequently, such as the effects of life stage, gender and pupal age (Wakid et al., 1976; Helinski et al., 2006, 2009; Balestrino et al., 2010; Akter and Khan, 2014) wheras very few, or only very old reports exist for the evaluation of others factors, such as effects of hypoxia or anoxia, temperature, and dose rate during radiation exposure (Hallinan and Rai, 1973; Curtis, 1976; El-Gazzar et al., 1983; Ernawan et al., 2017; Zhang et al., 2020).

More recently, a series of experiments to assess the impacts of several biological and physical factors (e.g., strain geographical origin, pupal age pupal size, atmospheric conditions) on dose-reponse in mosquitoes were conducted (Yamada et al., 2019; Yamada et al., 2020) with the aim to develop standardized protocols for the irradiation of mosquito pupae (FAO/IAEA, 2019). However; standardizing irradiation protocols for pupae is difficult, especially in practical terms in large-scale SIT programmes for the following reasons: Pupal age is an important factor that significantly impacts dose-response (Balestrino et al., 2010; Yamada et al., 2019). Although guidelines exist for the optimization of larval rearing for synchronized pupae development (FAO/IAEA, 2020), it is in reality unrealistic to narrow the pupation window to 16 h or shorter, to ensure that all pupae are aged 30 h or older during the irradiation process. Also, timing the pupation so that the collection, sexing and irradiation can occur during daytime working hours is another challenge. Irradiating mixed age batches is not recommended, as irradiating younger pupae can negatively affect adult quality (Balestrino et al., 2010), and over-dosing (as younger pupae require less dose) would further exacerbate this. Conversely, irradiating younger pupae at an optimal dose (to achieve >99% sterility) is possible, however the risk remains that older pupae would be under-dosed, leading to potentially releasing sub-sterile males, in addition to males with diminished quality, thereby compromising success of the otherwise effective SIT. Additionally, and equally problematic is that it is difficult, if not impossible to control the atmospheric conditions surrounding pupae during irradiation in bulk. For mass irradiation at the pupal stage, the pupae would need to be placed in sufficient water within the irradiation canister to provide buoyancy to avoid the pupae at the bottom being crushed. However, this creates a hypoxic environment as pupae submerged in water continue to respirate through their cuticle and quickly deplete the surrounding water of dissolved oxygen (Yamada et al., 2020). As hypoxia reduces irradiation effects, the irradiation of pupae in water results in differential levels of sterility within the sample (Yamada et al., 2020), therefore this method for irradiation cannot be reliable unless, again, the full cohort is significantly overdosed. Apart from quality costs of over-dosing, pupae exposed to hypoxia suffer additional stress and loss in quality. Large numbers of pupae can also be irradiated without water in monolayers, however, pupae are still closely packed and pockets of hypoxia still occur within the sample (Louis Clement Gouagna personal communication) resulting in a proportion of pupae maintaining unacceptable levels of fertility. Drying pupae and spreading them in a manner that would avoid these issues is simply not practical at large scale and is expected to incur detrimental levels of stress to the pupae.

For these reasons, the irradiation at adult stage could be a more practical and reliable option for the bulk sterilization of mosquitoes. Most notably, water, and thus hypoxia would no longer be a variable factor. Perfectly synchronized larval rearing (to achieve pupation within a 24 h window) would also no longer be as critical (and limiting) issue, significantly easing the practicality and efficiency of the irradiation process. However, to irradiate adult mosquitoes in bulk, these require immobiliaztion by either chilling (Culbert et al., 2019; Zhang et al., 2020) or treatment with anesthetics, such as in nitrogen, carbon dioxide, argon, chloroform, desflurane, or other alternative chemicals.

To verify the notion that standardizing irradiation for adult mosquitoes is feasible, we investigated some factors that may affect dose-response in adult male mosquitoes in comparison to pupae. Previous reports by Helinski et al. (Helinski et al., 2006) and Du et al. (Du et al., 2019) have shown that in both *Anopheles arabiensis* and *Aedes albopictus* respectively, adults are slightly more radio sensitive than old pupae, although the difference was generally not statistically significant. As no recent reports cover the comparative radiosensitivity of adults and old pupae in *Ae. aegypti*, we first studied the dose response curves of both life stages in this species, and then assessed the effects of ambient temperature (chilling), and anoxia in *Ae. albopictus* adults.

2 MATERIALS AND METHODS

2.1 Mosquito Strains and Rearing

Standard laboratory reference strains of *Ae. aegypti* and *Ae. albopictus* (FAO/IAEA, 2017, 2020) were used for all experiments. The *Aedes* strains have been maintained following the "Guidelines for Routine Colony Maintenance of *Aedes* mosquitoes" (FAO/IAEA, 2017).

2.2 Irradiation and Dosimetry

Radiation treatments were performed in a Gammacell 220 (Nordion Ltd., Kanata, Ontario, Canada), which had a dose-rate of 68 Gy/min during the temperature experiment (**Section 2.4**), and 65 Gy/min during the anoxia experiment (**Section 2.5**).

The dosimetry system used to verify the dose received by the samples was based on Gafchromic HD-V2 and MD-V3 film (Ashland Advanced Materials, Bridgewater NJ, United States) following the IAEA protocol (IAEA, 2004). Three films of either HD film (for doses >50 Gy) or MD film (for doses <50 Gy) were packed in small (2 × 2 cm) paper envelopes and placed directly above and below the mosquito samples. Films were read with an optical density reader after 24 h of development.

A diagnostic dose of 45 Gy was applied for most experiments, expecting to achieve around 95% sterility, to avoid 0 hatch results that cannot be usefully compared between treatments.

2.3 Assessing the Dose Response Curve for Pupal and Adult Stages of *Aedes aegypti*

Aedes aegypti were selected for this study as direct comparisons of pupal and adult radiosensitivity have not yet been reported in this species, contrary to *Ae. albopictus* (Du et al., 2019) and *An. arabiensis* (Helinski et al., 2006).

The doses for the dose-response curves for adult versus pupae of *Ae. aegypti* were selected according to the expected dose required to induce 50–100% sterility: 20, 55, 70, and 90 Gy.

Aedes aegypti eggs from one egg batch were collected and split in half to be hatched in two hatch events, 2 days apart (one for collecting adults, and one for collecting pupae for irradiation at the same time).

Adult males that emerged within an 8 h window were collected, batched in groups of 30, and kept in 15 × 15 × 15 cm Bugdorm® cages (MegaView Science Co. Ltd., Taichung 40762, Taiwan) until the following day when they were transferred to, and irradiated in small 2 cl plastic cups closed with a sponge. At the time of irradiation, the adults were 24–32 h old.

Pupae from the same cohort were collected in 4-h windows to ensure uniform pupal age of 40–44 h. We chose this age group as this represents the last hours before they begin to emerge into adults and are most radioresistant at this stage. The pupae were sexed based on pupal size dimorphism using a glass pupal sorter (Focks, 1980) and sex was verified under a stereomicroscope. Males were kept for treatment and females were placed in individual tubes for emergence to ensure virginity for later mating. Male pupae were counted into batches of 30 and were placed inside 2 cl plastic cups with excess water removed for irradiation.

Both the pupae and adults in each technical repetition were irradiated at the same time. Two biological repetitions and three technical repetitions were performed for all doses. Controls received the same handling but were not irradiated.

2.3.1 Assessment of Induced Sterility

Following irradiation, the male adults were placed in a 15 × 15 × 15 cm Bugdorm® cage, and pupae were placed in cups with water in separate cages for emergence. Thirty virgin females were added to each cage when the adults reached 2 days of age and were allowed to mate for 3 days before they were provided with 2 bloodmeals on consecutive days (days 6 & 7 post-emergence). Oviposition cups containing water and germination papers were added to each cage on day 8 for *en masse* egg collection (on days 9 & 10 post-emergence) following routine rearing protocols (FAO/IAEA, 2017). Egg papers were collected, matured (slow-dried over 4 days) and stored for 10 days before hatching. The total number of hatched and un-hatched eggs were counted using a stereomicroscope. Any non-hatched eggs were either opened with a dissection needle, or if many, were bleached to determine the fertility status (FAO/IAEA, 2019).

2.4 Effects of Chilling on Pupae and Adult Radiosensitivity, Flight Ability and Longevity in *Aedes albopictus* Irradiated as Adults

2.4.1 Dose-Response

As for the previous experiment, *Ae. albopictus* eggs from one egg batch were collected and split in half to be hatched in two hatch events, 2 days apart.

Adult males that emerged within an 8 h window were collected, batched in groups of 30, and were kept in 15 × 15 × 15 Bugdorm cages until the following day. The cages were then either kept at room (insectary) temperature (27° ± 2°C) ("rm temp") or were placed in a cold room for knock down at 5°C for 5 min, and then in a climatic chamber at 7°C ("chilled") for 1 h prior to the irradiation event. The treatments were thus either Control rm temp, or 45 Gy rm temp, or control chilled, or 45 Gy chilled. The adult males for the irradiation treatment were then transferred to 2 cl plastic cups closed with a sponge and were taken to the irradiator in Styrofoam boxes; the chilled males were kept in the cool box at 7°C until placed inside the GC220

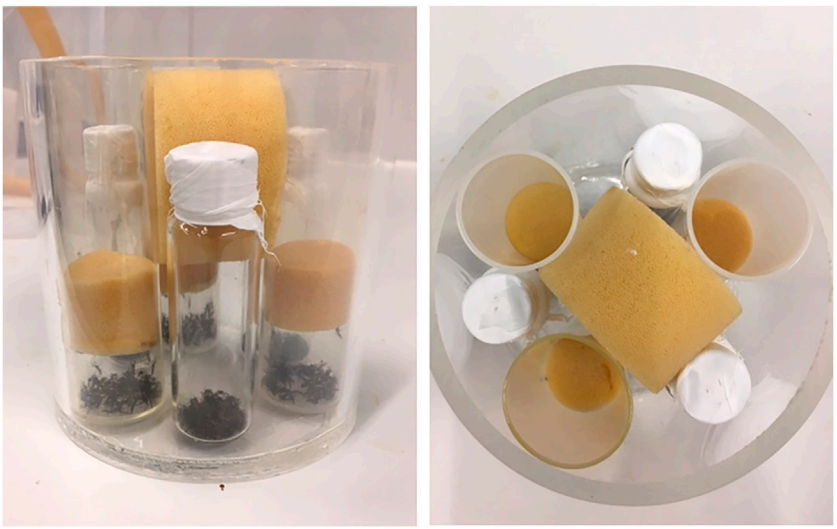

FIGURE 1 | Irradiation set-up for adults irradiated in nitrogen or in air. One biological repetition with each three technical repetitions were irradiated simultaneously for each treatment, within a 5 mm thick PMMA container. The container was placed on a Styrofoam step so that all samples were in the middle of the irradiation chamber.

irradiator chamber and irradiated in same small 2 cl plastic cups closed with a sponge. At the time of irradiation, the adults were 2 days old.

Pupae from the same cohort were collected in an 8-hour window to ensure that all pupae were at least 36 h old during irradiation. The pupae were sexed based on pupal size dimorphism using a glass pupal sorter (Focks, 1980) and sex was verified under a stereomicroscope. Males were kept for treatment and females were placed in individual tubes for emergence to ensure virginity for later mating for both the adults and pupae treatment groups. Male pupae were counted into batches of 30 and were placed inside 2 cl plastic cups. The samples were subjected to the same treatments as described for adults above. The treatments were thus either Control rm temp, or 45 Gy rm temp, or control chilled, or 45 Gy chilled. Before irradiation, excess water was removed from the cups holding pupae. Two biological repetitions with each 3 technical repetitions were performed for each treatment.

Egg hatch rates were assessed as described in the above section "Assessing the dose response curve for pupal and adult stages following irradiation in a GC220/2.3.1 Assessment of induced sterility".

2.4.2 Longevity Under Mating Stress

Three of each treatments group and controls each, for both "adults" and "pupae" were kept in the cages post mating and oviposition to follow the longevity of the males. Dead males were counted and removed at least 4 times per week until all males were dead.

2.4.3 Flight Ability

One hundred male adults per treatment (rm temp or chill) and per repetition were collected from the same cohort and irradiated as described in the above section "dose response".

After irradiation, adults were allowed to recover for 2 days before they were taken to the flight test devices. Each batch of 100 males were placed inside the flight tubes for a duration of 2 h. Escaped and non- escaped adults were then counted as described by Culbert et al. (Culbert et al., 2018). Two repetitions with each 2 technical repetitions were completed.

2.5 Effects of Anoxia on Adult Dose-Response, Flight Ability and Longevity in *Aedes albopictus*

2.5.1 Dose-Response

Adult *Ae. albopictus* males that emerged within an 8 h window were collected, batched in groups of 20–30, and were kept in 15 × 15 × 15 cm Bugdorm cages until the following day. The batches of adult males for the "normoxic treatment" were then transferred to plastic "*Drosophila*" tubes (9 cm height × 2.7 cm diameter) closed with a sponge. The sponges were pushed down before irradiation so that the samples were in a similar position to the adults immobilized with nitrogen (**Figure 1**). Adult batches for the "anoxic treatment" were placed in gas tight glass head space vials (20 ml) with screw tops with PTFE/silicon septum (Merck KGaA, Darmstadt, Germany), additionally sealed with PTFE Thread Seal Tape (Sigma-Aldrich, United States). The oxygen was then replaced by nitrogen by adding nitrogen *via* a syringe needle (a second needle was inserted for outgoing gas), for 10 s, until all adult mosquitoes were immobile, and the 2 syringe needles were removed. Both anoxic and normoxic groups (3 technical repetitions each) were irradiated at 45Gy simultaneously (in alternating positions) in a 12 cm diameter PMMA container, in the GC220 irradiator (**Figure 1**). Three biological repetitions from different cohorts (with each 3 technical repetitions) were performed in total. At the time of irradiation, the adults were 2 days old.

Induced sterility was assessed as described in the above section "Assessing the dose response curve for pupal and adult stages following irradiation in a GC220/2.3.1 Assessment of induced sterility".

2.5.2 Flight Ability

One hundred male adults per treatment (normoxia (oxygen) or anoxia (nitrogen), irradiated at 45 Gy, and non-irradiated controls) and per repetition were collected from the same cohort and irradiated as described in the above section "dose-response".

Flight tests were performed as described in the above section "**Section 2.4** and **Section 2.4.3**". Two repetitions with each 3 technical repetitions were completed.

2.5.3 Longevity Under Mating Stress

All treatments groups and controls were kept in the cages post mating and oviposition to follow the longevity of the males. Dead males were counted and removed at least 4 times per week until all males were dead. Three repetitions were done for all treatment groups and controls for both treatment groups irradiated as pupae and as adults.

2.5.4 Longevity Following High Doses- Males Only

As little difference was seen following the previous longevity experiments, and mating stress is known to decrease survival in males, an additional experiment was added to assess the effects of anoxia on sterile male longevity, without the added factor of mating stress. For this, additional batches of 30 males were irradiated in either normoxia (oxygen) or anoxia (nitrogen) as described in the previous section. All males were over-dosed at 90 Gy (beyond the fully sterilizing dose) in the GC220 as described above. The males were then returned to 15 × 15 × 15 cm Bugdorm cages and dead males were counted and removed at least 4 times per week until all males were dead.

2.6 Statistical Analysis

All statistical analyses were performed in R (version 4.1.0) using RStudio (RStudio, Inc. Boston, MA, United States, 2016). Generalized Linear Mixed Models (GLMM, lme4 package) were used with the appropriate distribution family.

To analyze the dose response curve of pupae versus adults for *Ae. aegypti*, a binomial GLMM fit by maximum likelihood (Laplace Approximation) was used for egg hatch rates considered as response variable, life stage (2 levels: pupae and adults), irradiation log (dose) (4 levels: 20, 55, 70 and 90 Gy) and their interaction were considered as fixed effects and the repetition as a random effect.

For the effects of chilling on pupae and adult radio-sensitivity in *Ae. albopictus*, a binomial GLMM was also used with egg hatch rates as response variable, treatment (2 levels: room, chilling), life stage (2 levels: pupae and adults), irradiation dose (2 levels: 0 and 45 Gy) and their interaction considered as fixed effects and repetition nested with technical repetition as a random effect.

Similarly, male flight ability data was analyzed as response variable, treatment (4 levels: Chilled/room temperature, irradiated/non-irradiated; or anoxia/normoxia and irradiated/non-irradiated) as fixed effect and the repetition nested with technical repetition as a random effect considering each specific experiment.

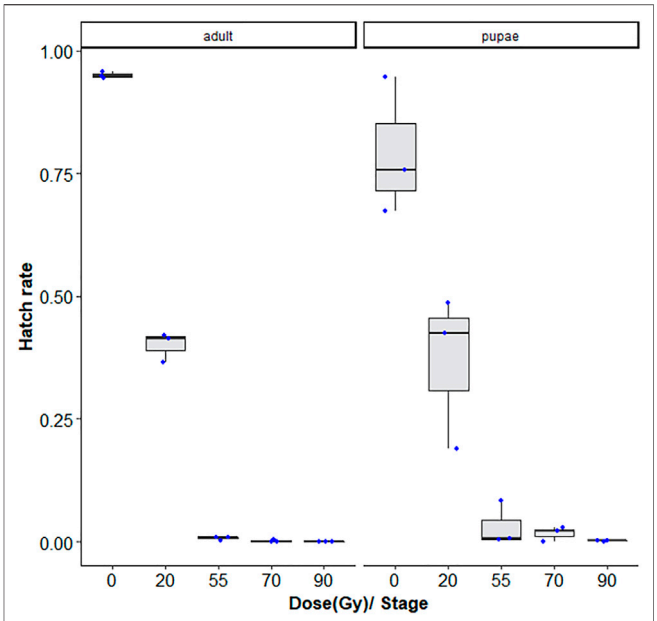

FIGURE 2 | Dose-response shown as egg hatch rate of *Aedes aegypti* pupae vs. adults. The boxplot shows the median, and upper and lower quartiles. The dots indicate the values obtained for each repetition.

Mixed Effects Cox Models ("coxme" function in 'survival' package) fit by maximum likelihood with mosquito time to death as response variable, treatment (4 levels: chilled, room temperature, irradiated, non-irradiated; or 3 levels: anoxia, normoxia, non-irradiated control) and their interaction as fixed effects and repetition as random effect, were used to analyze the survival of mosquitoes following the treatment in each specific experiment. Survival graphs were built using the packages "survival," "ggplot2," and "ggpubr".

The full models were checked for overdispersion using Bolker's function (Bolker and R Development Core Team, 2020) (in package bblme). The best model was chosen based on the lowest AICc s and models were simplified using the stepwise removal of terms, followed by likelihood ratio tests (LRTs) when appropriate. Multiple comparisons using the "emmeans" function (in package "emmeans") (https://github.com/rvlenth/emmeans) were performed between the levels where significant differences were found. A p-value of less than 0.05 was used to indicate statistical significance in all cases.

3. RESULTS

3.1 Dosimetry

The dosimetry confirmed that all doses received laid within a 5% error range.

TABLE 1 | Fixed effects of chilling on pupae and adult radiosensitivity.

	Estimate	Std. Error	z value	Pr (>|z|)
(Intercept)	2.0095	0.1826	11.005	<2e-16 ***
Room temperature	−0.3408	0.1342	−2.539	0.0111 *
Pupae stage	0.2332	0.1315	1.773	0.0762
Dose45Gy	−4.6976	0.1651	−28.446	<2e-16 ***

*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05 "." 0.1 " " 1.*

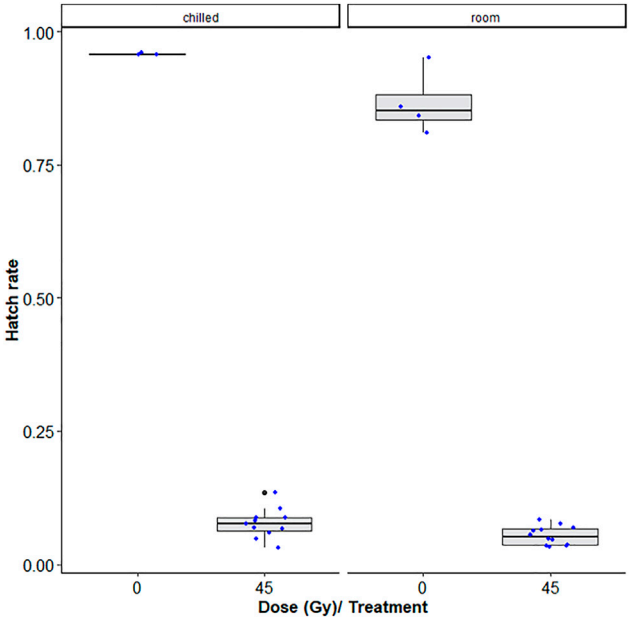

FIGURE 3 | Dose-response shown as egg hatch rate of *Aedes aegypti* pupae vs. adults. The boxplot shows the median, and upper and lower quartiles. The dots indicate the values obtained for each repetition.

3.2 Assessing the Dose Response Curve for Pupal and Adult Stages of *Aedes aegypti*

As expected, the hatch rate reduced signficantly with the dose (GLMM: χ^2 = 2,589, df = 2, $p < 0.001$). For 20, 55, 70, and 90 Gy doses tested, adults were more radiosensitive than the late stage pupae, with <1–~3% lower fertiliy levels following radiation exposure (GLMM: χ^2 = 52.685, df = 2, $p < 0.001$, **Supplementary Table S1**). There was also a higher degree of variation observed between repetitions for the pupae samples (**Figure 2**).

3.3 Effects of Chilling on Pupae and Adult Radiosensitivity, Flight Ability and Longevity in *Aedes albopictus* Irradiated as Adults

3.3.1 Dose-Response

Table 1 shows that room temperature led to lower hatch rates as compared to chilling, i.e., chilling led to decreased induced sterility (GLMM: χ^2 = 6.4454, df = 1, p = 0.0111, **Figure 3**) while no difference was observed between pupae and adult *Ae. albopictus* mosquito stages (χ^2 = 3.1432, df = 1, p = 0.07625). Additionally, irradiation dose of 45Gy significantly reduced the egg hatch rates (χ^2 = 809.1845, df = 1, $p < 0.001$, **Figure 3**).

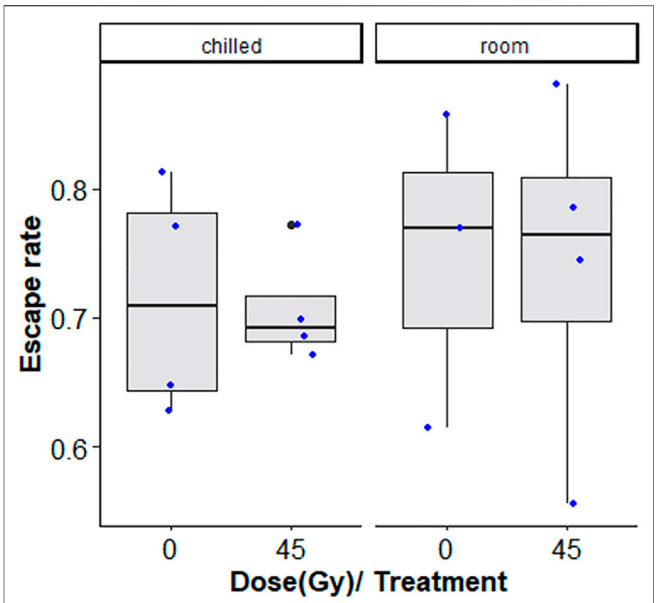

FIGURE 4 | Flight ability of chilled versus non-chilled, sterile *Aedes albopictus* male adults irradiated at 45 Gy or not irradiated The boxplot shows the median, and upper and lower quartiles. The dots indicate the values obtained for each repetition.

TABLE 2 | Fixed effects of chilling and irradiation on flight ability in *Aedes albopictus males*.

	Estimate	Std. Error	z value	Pr (>|z|)
(Intercept)	0.85361	0.09806	8.705	<2e-16 ***
Room temperature	0.15859	0.10535	1.505	0.132
Dose45Gy	0.04216	0.09957	0.423	0.672

*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05 "." 0.1 " " 1.*

TABLE 3 | Fixed coefficients of the effects of pupae chilling on male *Ae. albopictus* longevity.

	Coef	exp (coef)	se (coef)	z	*p*
Dose	0.001195	1.001196	0.004363	0.27	0.78
Doom temperature	0.05391	1.055389	0.24482	0.22	0.83
Dose:room temperature	−0.003	0.997009	0.006248	−0.48	0.63

*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05 "." 0.1 " " 1*

3.3.2 Effects of Chilling on Flight Ability

Chilling for 1 h at 7°C, with or without irradiation at 45 Gy followed by 2-day-recovery had no negative effects on flight ability (**Figure 4**; **Table 2**).

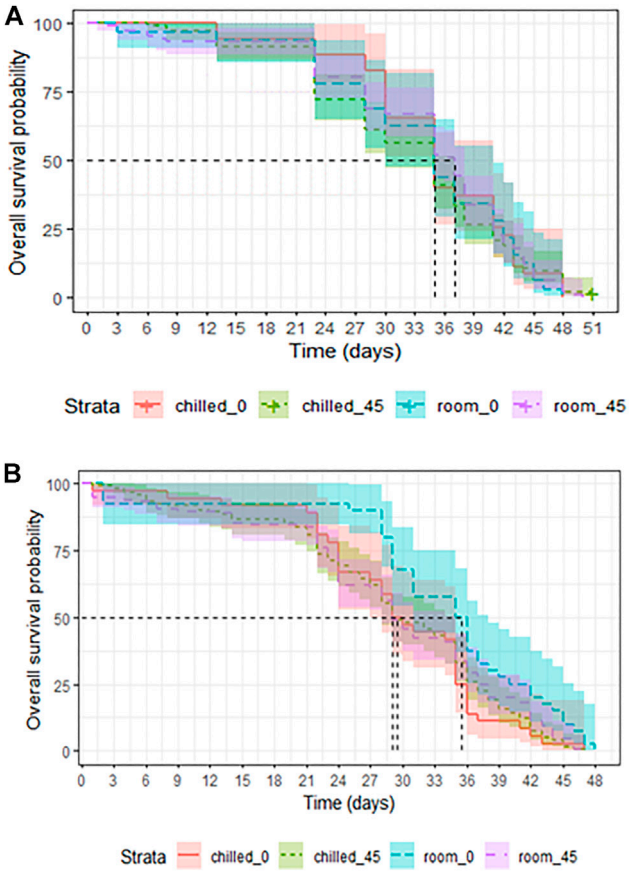

FIGURE 5 | Longevity of chilled versus non-chilled *Aedes albopictus* males irradiated at 45 Gy as either pupae **(A)** or as adults **(B)**, compared to non-irradiated controls.

TABLE 4 | Fixed coefficients of the effects of adult chilling on male *Ae. albopictus* longevity.

	coef	exp (coef)	se (coef)	z	p
Dose45Gy	0.210645	1.234474	0.138563	1.52	0.13
Room temperature	−0.19772	0.820597	0.11508	−1.72	0.086

*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05 "." 0.1 " " 1.*

3.3.3 Effects of Chilling on Male Longevity

In adults that were chilled and/or irradiated as late pupae, survival was not affected by chilling (LRT: $\chi^2 = 0.1582$, df = 1, $p = 0.6908$, **Table 3**), nor by irradiation with 45Gy (LRT: $\chi^2 = 0.0006$, df = 1, $p = 0.9383$, **Table 3**). Adults that were chilled and irradiated as pupae also lived as long as untreated controls (LRT: $\chi^2 = 0.224$, df = 1, $p = 0.6317$, **Table 3** and **Figure 5A**).

In mosquitoes that were chilled and/or irradiated at the adult stage, chilling had no negative effect on the overall longevity of adults (LRT: $\chi^2 = 2.947$, df = 1, $p = 0.08603$, **Table 4**). Irradiation at 45 Gy also did not affect overall longevity as compared to non-irradiated adults (LRT: $\chi^2 = 2.2893$, df = 1, $p = 0.1303$, **Table 4**). Adults that underwent chilling plus irradiation at 45 Gy were marginally negatively impacted as compared to untreated controls (LRT: $\chi^2 = 3.39$, df = 1, $p = 0.06559$, **Figure 5B**). Chilling, and combined chilling and irradiation treatments reduced the median survival from the 35.5 (31-38, 95%CI) days in the control group to 29.0–29.5 (28-35, 95%CI) days (**Supplementary Table S2**), however, the difference was not significant.

TABLE 5 | Fixed effect of anoxia on adult dose-response.

	Estimate	Std. Error	z value	Pr (>|z|)
(Intercept)	4.3298	0.1959	22.105	<2e-16 ***
Dose45Gy	−5.2631	0.1317	−39.954	<2e-16 ***
Normoxia	−1.0435	0.1425	−7.323	2.43e-13 ***
Dose45Gy:rormoxia	−0.8462	0.1546	−5.474	4.39e-08 ***

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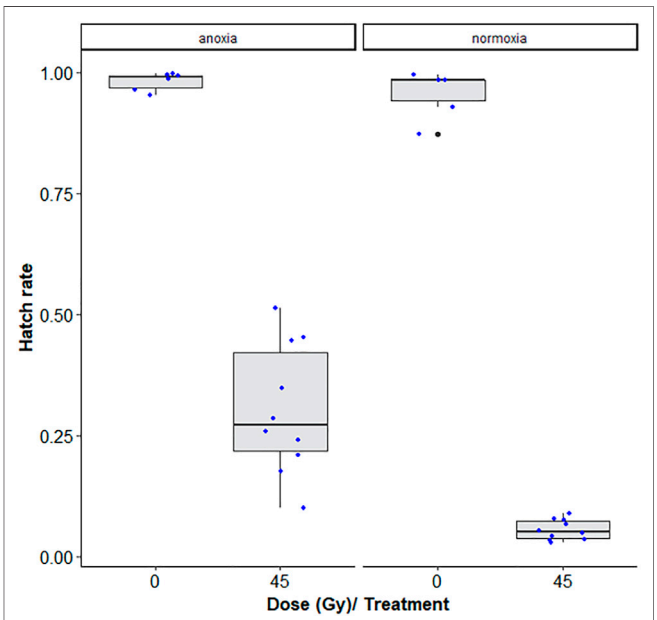

FIGURE 6 | Egg hatch rates of *Aedes albopictus* males irradiated at 45Gy in normoxia (O_2) vs. anoxia (N_2). The boxplot shows the median, and upper and lower quartiles. The dots indicate the values obtained for each repetition.

3.4 Effects of Anoxia on Adult Dose-Response, Flight Ability and Longevity in *Aedes albopictus*

3.4.1 Dose-Response

There was a significant interaction between anoxia and irradiation dose effects ($\chi^2 = 29.968$, df = 1, $p < 0.001$, **Table 5**). Adult males irradiated at 45 Gy in anoxia were on average 5.7 times more fertile than those irradiated in normoxia ($p < 0.001$, **Supplementary Table S3**; **Figure 6**). The highest observed difference in fertility was a 14-fold difference between samples irradiated in anoxia versus normoxia. More variability

TABLE 6 | Fixed effects of irradiation in anoxia on flight ability.

	Estimate	Std. Error	z value	Pr (>|z|)
(Intercept)	1.922925	0.151896	12.66	<2e-16 ***
Atmnormoxia	0.605234	0.178105	3.398	0.000678 ***
Dose45Gy	−0.00993	0.177462	−0.056	0.955386

*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05 "." 0.1 " " 1.*

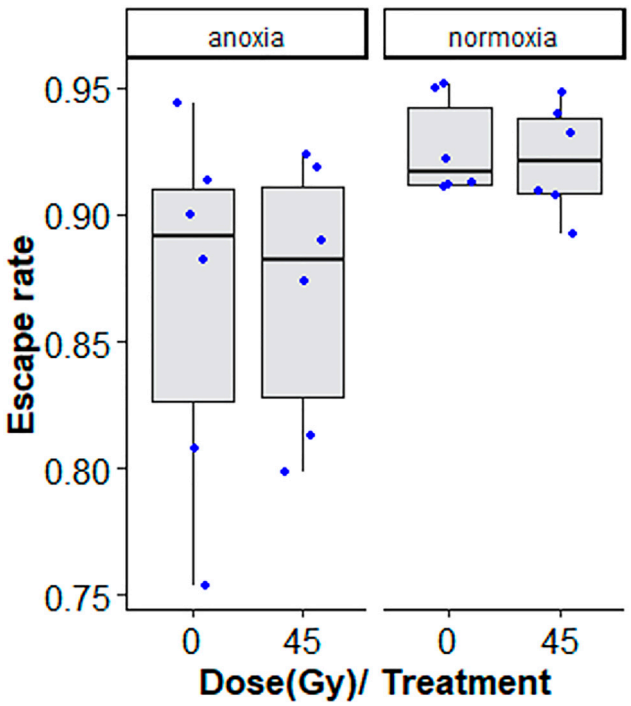

FIGURE 7 | Flight ability of adults treated in nitrogen [non-irradiated controls (N_2C) and irradiated (N_{2i})] compared to adults in air [non-irradiated controls (O_2C) and irradiated in air (O_{2i})]. The boxplot shows the median, and upper and lower quartiles. The dots indicate the values obtained for each repetition.

within samples and between technical repetitions were also observed in the anoxia treated groups as compared to the normoxic groups.

3.4.2 Effect of Irradiation in Anoxia on Flight Ability

Treatment with nitrogen (anoxia) negatively affected flight ability, regardless of whether irradiated or non-irradiated (GLMM: χ^2 = 29.642, df = 1, p < 0.001, **Table 6** and **Supplementary Table S4**). Irradiation at 45 Gy did not reduce flight ability, neither in the anoxia treatment groups), nor in the normoxic (oxygen) groups (GLMM: χ^2 = 0.0829, df = 1, p = 0.7734, **Figure 7**; **Supplementary Table S4**). Again, results were much more variable in groups subjected to anoxia.

3.4.3 Longevity Under Mating Stress

A significant interaction between dose and atmosphere was observed (LRT: χ^2 = 19.427, df = 1, p < 0.001). However, when

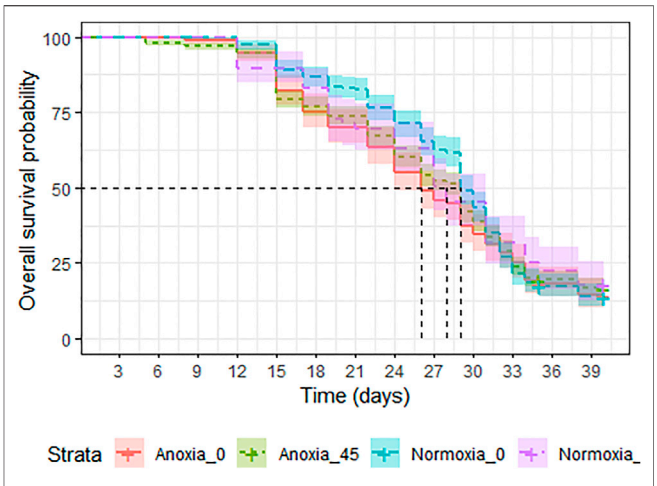

FIGURE 8 | Longevity under mating stress of adult *Aedes albopictus* males irradiated at 45 Gy in either anoxia (N_2) or normoxia (O_2) compared to untreated controls.

comparing each treatment group, anoxia had a negative effect on survival in the non-irradiated groups (Odd ratio = 1.269, z. ratio = 2.652, p = 0.04, **Supplementary Table S5**), but decreased the risk of mortality in the irradiated groups (Odd ratio = 0.631, z. ratio = −3.793, p = 0.0009, **Supplementary Table S5**), when males were caged with females at a 1:1 ratio and were assessed under mating stress (**Figure 8**). Between the normoxic groups, irradiation with 45 Gy reduced the longevity slightly (Odd ratio = 0.593, z. ratio = −4.146, p = 0.0002, **Supplementary Table S5**). Median survival was 29 (95%CI 29–30) days for untreated controls, and for the goups irradiated in anoxia (95%CI 27–29). Groups irradiated in normoxia and groups treated with only anoxia showed a slightly reduced median survival time of 28 (95%CI 27–31) and 26 (95%CI 27–29) days, respectively (**Supplementary Table S6**).

3.4.4 Longevity Following High Doses- Males Only

Treatment had a significant effect on adult survival (LRT: χ^2 = 164.9, df = 2, p < 0.001). Longevity of males irradiated in anoxia was not affected compared to untreated controls (p = 0.054, **Table 7**; **Figure 9**) even when the dose was doubled to 90 Gy, wheras males irradiated in normoxia at the same dose were highly negatively impacted (p < 0.0001, **Table 7**; **Figure 9**). The median survival of the untreated controls and the adults irradiated with 90 Gy in anoxia was 43 (95% CI 40–43) and 41 (95%CI 41-41) days respectively, wheras adults irradiated with 90 Gy in normoxia had a median survival of 28 (95%CI 28-28) days (**Supplementary Table S7**).

4 DISCUSSION

The series of experiments reported here have shown that there are various factors that affect dose response in mosquito adults, which need to be taken into consideration when developing

TABLE 7 | Fixed coefficients of the effects of anoxia with high doses on longevity for males only.

	coef	exp (coef)	se (coef)	z	p
Anoxia_90Gy	−0.19949	0.819147	0.103508	−1.93	0.054
Normoxia_90Gy	1.034523	2.813763	0.102299	10.11	0

*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05 "." 0.1 " " 1.*

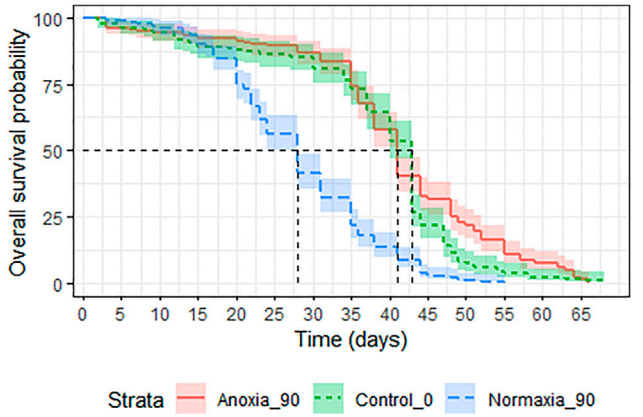

FIGURE 9 | Longevity of adult male *Aedes albopictus* irradiated at high dose (90 Gy) in normoxia (O_2) or anoxia (N_2) compared to untreated controls.

irradiation protocols for adults in the frame of the SIT. These factors not only affect irradiation outcome in terms of sterility levels achieved in the males, but also downstream quality parameters important for male performance once released.

Adult *Ae. aegypti* mosquitoes were slightly more radiosensitive than late-stage pupae. However, one must consider that the younger the pupae, the more sensitive and the more prone to somatic damage (Yamada et al., 2019). In general, *Aedes* pupae, just before emergence seem to be at their most resistant phase to various treatments, such as chilling, desiccation, hypoxic environments and irradiation contrarily to (some) fruit flies, where the pupae are most sensitive on the day before emergence when they are undergoing extensive mitotic divisions for the buildup of the adult organism (Economopoulos, 1977). The dose-response curves for pupae versus adult *Ae. aegypti* corroborate those reported for *An. arabiensis* (Helinski et al., 2006) and *Ae. albopictus* (Du et al., 2019), where adults were slightly more sensitive, but for the most part, the difference was not significant, as was seen in the comparison of late-stage pupae and adult *Ae. albopictus* in the second experiment. The homogeneity of sterility levels within and between adult samples was better than in pupae samples, although pupal age was carefully controlled. Considering that adults do not require higher doses, and the high levels of consistency seen within and between irradiated batches are advantages of irradiating at this stage, in addition to facilitated timing and ease of handling for irradiation exposures.

The next factor that requires scrutiny is the immobilization of adults during bulk irradiation needed for operational programmes. Therefore, assessing the effects of chilling on dose-response in terms of sterility and downstream male performance was essential. Many studies are available in which cold temperatures reduced insect flight ability, or mating competitiveness (Mutika et al., 2002; Shelly et al., 2010; Reynolds and Orchard, 2011; Andress et al., 2014; Diallo et al., 2019), or where cold treatment was used to enhance phytosanitary practices (Gould and Sharp, 1990; von Windeguth and Gould, 1990; Burikam et al., 1992; Follett and Snook, 2013; de Kock and Holz, 2017). However, in these reports, cold treatments were applied separately from the irradiation step, and the direct effect of chilling on dose response was not investigated.

Andress et al., (Andress et al., 2012) found that chilling (3–8°C for 2–6 h) decreased flight ability dramatically in the Mediterraenan fruitfly (*Ceratitis capitata*), whereas Tanahara and Kirihara (Tanahara and Kirihara, 1989), and Reynold and Orchard (Reynolds and Orchard, 2011) found no detrimental effects of chilling on this parameter in the melon fly (*Bactrocera curcurbitae*) and Qeensland fruitfly (*B. tryoni*) respectively. Shelly et al. (Shelly et al., 2010) observed negative effects of chilling on the flight capacity and mating performance of *C. capitata* held at high densities, however, the mating performance was restored after 3 days of recovery. A recovery of host-searching abilities of the parasitoid *Diachasmimorpha longicaudata* following damages from chilling for packaging was also observed after 1–2 days (Cancino et al., 2020). In tsetse flies, Diallo et al. (2019) reported that chilling was one of the main factors negatively affecting the quality of the sterile flies, in terms of emergence rates and flight ability. This corroborated findings of Mutika et al. (2002), who reported a decrease in insemination rates and a dramatic increase in mortality of adult *Glossina pallipides* following low temperature (7 and 4°C)treatment of pupae.

There are numerous other studies that evaluate the effects of temperature on insect quality, however few exist that assessed cold temperature effects during radiation exposures on the irradiation outcome itself. Most studies investigated its direct effects on fertility, or the downstream effects of the combined irradiation and chilling on sterile male quality. For instance, a decrease in survival of adult *G. morsitans* was reported as irradiation dose was increased, and this decline was more pronounced in cohorts irradiated at low temperatures (2°C) (Curtis and Langley, 1972). Langley and Maly (1971) also found chilling to have deleterious effects on adult emergence rates and adult male survival in the fruitfly *C. capitata*, and proposed oxygen-dependent effects of irradiation to be the cause, due to higher oxygen saturation levels at lower temperatures.

Chilling *Ae. albopictus* at 7°C before and during irradiation had no negative impact on longevity when treated as pupae, and only resulted in a marginal reduction in longevity when treated as adults (though not statistically significant). However, the chilling conditions of the present study did slightly reduce the sterility levels achieved as compared to males that did not undergo chilling treatments, but only by a few percent (~3%). This implies that the cold temperature had some degree of radioprotective effect during the exposure. Here, it was again observed that the variation in sterility levels was higher in and

between pupae samples and adult samples were more homogeneous, as was seen with *Ae. aegypti* in the first experiment in this report.

Culbert et al. (2019) studied the effects of chilling in mosquito adults on quality control (QC) parameters, and found that chilling had negative effects on the survival in *Ae. aegypti* and *Ae. albopictus*, where the latter was more sensitive to the cold treatment at all tested temperatures. Contrarily, chilling (at 2,4,6 and 10°C) for up to 8 h had no effect on the survival of *An. arabiensis* for 14 days. Only chilling at 2°C for 24 h resulted in a decrease in longevity in this species. Zhang et al. (2020) found the optimum chilling temperature and duration for *Ae. albopictus* to be 5–10°C for 3 h, resulting in no adverse effects on longevity and mating competitiveness.

Following a similar trend, it was found that chilling did not significantly reduce flight ability, although chilled irradiated groups showed marginally reduced escape rates (though not significant), compared to the unirradiated controls. There was no significant difference between chilled and non-chilled, unirradiated controls, due to a high variation in escape rates in both groups. It is possible that sufficient recovery of chilled adults occurs within the 2 days before the flight test. Significant decrease in flight ability was seen directly after chilling, but near full recovery was observed after 1–2 days in *Ae. aegypti* (Maiga, unpublished results).

The slight reduction in the sterility following irradiation in cold temperatures may be the consequence of the reduced metabolic rates in the mosquitoes, whereas the slight increase somatic damage leading to marginally reduced adult quality in the parameters assessed, and chilling induced damages as reported in other studies (referenced above) may be explained by higher oxygen saturation in the low temperature, leading to an increase in oxygen-dependent effects of irradiation, as proposed by Langely and Maly (Langley and Maly, 1971). In any case, it is known that both radiation damage as well as recovery are temperature-dependent and are both slow in cold temperatures (Sazykina and Kryshev, 2011).

There is a threshold for different insect species at which cold temperatures start to cause negative effects on the organism. For this reason, available studies present either negative or no effects, and seldom positive effects regarding sterile insect quality in the frame of the SIT. The slight radioprotective effects of irradiation in cold temperatures as seen here does not present added value in terms of improving mosquito quality or the SIT, other than its practicality of immobilizing and handling the adult mosquitoes. On the contrary, the degree of chilling and duration is important as it can induce negative effects if not controlled carefully. Therefore, it is worth investigating other methods for immobilization that may improve sterile male quality and irradiation procedures.

Nitrogen can also be used to immobilize mosquitoes for handling and irradiation processes, and its protective effects in insect irradiation have been known since 1947 (Thoday and Read, 1947) and has been widely reported for a variety of insect species.

Hypoxic conditions during insect irradiation have also been shown to often improve insect biological quality, even though higher doses are then needed to reach the desired sterility levels (Economopoulos, 1977; Ohinata et al., 1977; Rananavare et al., 1991). This is because the magnitude of the protective effects seems to be greater for somatic damage than for the induction of sterility (Bakri et al., 2021). For this reason, hypoxic conditions are often used to improve sterile insect quality without reducing their sterility levels (Lance and McInnis, 2005). However, some agents used to create hypoxic environments, such as CO_2 and N_2 are reported to have their own negative side effects, some of which disappear again after allowing a period of recovery (Birkenmeyer and Dame, 1970), and some with lasting effects. Other studies on irradiation in anoxia report great improvements on several quality parameters such as longevity, developmental parameters and mating performance (Baldwin and Salthouse, 1959; Baldwin and Chance, 1970; Langley and Maly, 1971; Curtis and Langley, 1972; LaChance and Richard, 1974; Fisher, 1997).

Only few reports exist where irradiation of mosquitoes in nitrogen is described, and effects on quality parameters are assessed. El-Gazzar (1983) exposed *Culex quinquefasciatus* to radiation in a nitrogen atmosphere and showed the reduced effects on sterility induction but found little to no improvement on mating performance. Hallinan and Rai (Hallinan and Rai, 1973) reported that for low doses, nitrogen improved mating competitiveness in *Ae. aegypti*, compared to males irradiated in air, similar to what Terwedow and Asman (Terwedow and Asman, 1977) reported for *Ae. sierrensis*, but none of the publications describe any improvement in other male quality parameters.

Our study also showed that hypoxia protects from O_2 effects during adult irradiation as was seen in mosquito pupae (Yamada et al., 2019, 2020), but may come with its own negative effects. Anoxia had high radioprotective effects, with up to a 14-fold increase in residual fertility compared to males irradiated with the same dose in normoxia. Anoxia did not have an effect on fertility in unirradiated controls. However, the treatment with N_2 itself had a negative impact on flight ability. The 2-day recovery time allowed males that were irradiated only, to fully recover flight ability, whereas those treated in N_2 were unable to recover within this time frame, whether irradiated or not. This implies that the treatment with N_2 was more important for the reduced flight capacity than the irradiation treatment with 45 Gy. It is possible that a longer recovery time could restore flight ability but storing sterile males for much longer than 2–3 days post irradiation may decrease efficiency in the SIT programmes, where space and extra days of handling are costly.

There was a slight reduction in longevity when adult males were irradiated with 45 Gy in normoxia or anoxia, when they were caged with females at a 1:1 ratio. However, when males were caged alone, and were not subjected to mating stress, those males overdosed with 90 Gy in anoxia survived significantly better than the males irradiated with the same dose in normoxic conditions. We suggest that either 45 Gy is a low enough dose in this species to not see a large effect on longevity [as seen in other reports (Balestrino et al., 2010)], or males irradiated in anoxia are not only more fertile, but also more virile, mating more, and thus slightly reducing longevity in the mixed sex cages, contrary to the results seen at the higher dose, but where females were absent. Males overdosed with 90 Gy in nitrogen lived significantly longer

that males irradiated in oxygen and the protective effects of anoxia were clearly observed. To better understand the meaningfulness of longevity studies, it would be important to further examine the effects of the various study designs and variables, such as cage size and adult density, and the inclusion of females (at various ratios) to observe the magnitude of mating induced stress and its effects on male survival. In this study, the males caged alone generally survived more than 2 weeks longer than the males caged with females, suggesting that mating stress has considerable effects on survival and can mask effects of other treatments that are actually the focus of the study.

Although nitrogen had radio protective effects which may preserve fertility and longevity, it seems that treatment with nitrogen in general (with or without the additional radiation exposure) had negative effects on male flight ability, and potentially other parameters which may be the more important factors for mating success in the wild. The full effects of anoxic treatments need to be carefully assessed in field cage mating studies.

There is a need for anesthetics for insect immobilization for facilitating handling. However, many reports have shown that immobilizing agents induce negative side effects (Crystal, 1967; Birkenmeyer and Dame, 1970), the extent of which depends on the sex and age of the insect, as well as the duration and frequency of exposures to the various gases, similar to treatments in cold temperatures. It is therefore necessary to carefully assess these factors and all available options before formulating protocols for mosquito immobilization and handling.

5 CONCLUSION

These experiments gave an initial indication of factors that affect dose-response in mosquito adults, especially in terms of sterility achieved, and downstream effects of chilling and anoxia on selected male quality parameters. Both methods present advantages and disadvantages and affect some quality parameters positively and others negatively. It is important to note that the irradiation dose needs to be adjusted to achieve the desired level of sterility, before considering treatment protocols that could improve sterile male quality for SIT programmes. Additionally, available immobilizing techniques for improved handling need careful evaluation and balance between practicality and potential costs to insect quality to ensure there is a clear benefit before their application in the field.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

HY conceptualized the experimental designs for the experiments, caried out the experiments and drafted the original manuscript. HM carried out the flight tests and contributed significantly to the written content and later versions of the manuscript. AA contributed to the design of the anoxia setup and experiment and provided equipment and materials. CK, WM, NB, and TW provided all live material following standardized rearing procedures and assisted in the experiment and data collection. JB and HM contributed to the experimental designs and carried out the statistical analyses. JB supervised and supported the project. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.856780/full#supplementary-material

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Radiation dose-fractionation in adult *Aedes aegypti* mosquitoes

Hanano Yamada*, Hamidou Maïga, Carina Kraupa, Nanwintoum Séverin Bimbilé Somda, Wadaka Mamai, Thomas Wallner, and Jeremy Bouyer

Insect Pest Control Laboratory, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, PO Box 100, 1400 Vienna, Austria

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Abstract – Balancing process efficiency and adult sterile male biological quality is one of the challenges in the success of the sterile insect technique (SIT) against insect pest populations. For the SIT against mosquitoes, many stress factors need to be taken into consideration when producing sterile males that require high biological quality to remain competitive once released in the field. Pressures of mass rearing, sex sorting, irradiation treatments, packing, transport and release including handling procedures for each step, add to the overall stress budget of the sterile male post-release. Optimizing the irradiation step to achieve maximum sterility while keeping off-target somatic damage to a minimum can significantly improve male mating competitiveness. It is therefore worth examining various protocols that have been found to be effective in other insect species, such as dose fractionation. A fully sterilizing dose of 70 Gy was administered to *Aedes aegypti* males as one acute dose or fractionated into either two equal doses of 35 Gy, or one low dose of 10 Gy followed by a second dose of 60 Gy. The two doses were separated by either 1- or 2-day intervals. Longevity, flight ability, and mating competitiveness tests were performed to identify beneficial effects of the various treatments. Positive effects of fractionating dose were seen in terms of male longevity and mating competitiveness. Although applying split doses generally improved male quality parameters, the benefits may not outweigh the added labor in SIT programmes for the management of mosquito vectors.

Key words: Irradiation, Induced sterility, Flight ability, Competitiveness, Rhodamine B, Sterile insect technique.

Résumé – Fractionnement de la dose d'irradiation chez les moustiques *Aedes aegypti* adultes. Équilibrer l'efficacité du processus et la qualité biologique des mâles adultes stériles est l'un des défis du succès de la technique des insectes stériles (TIS) contre les populations d'insectes nuisibles. Pour la TIS contre les moustiques, de nombreux facteurs de stress sont à prendre en compte lors de la production de mâles stériles qui nécessitent une haute qualité biologique pour rester compétitifs une fois relâchés au champ. Les pressions de l'élevage en masse, du triage par sexe, des traitements d'irradiation, de l'emballage, du transport et de la libération, y compris les procédures de manipulation pour chaque étape, s'ajoutent au budget de stress global du mâle stérile après la libération. L'optimisation de l'étape d'irradiation pour atteindre une stérilité maximale tout en minimisant les dommages somatiques hors cible peut améliorer considérablement la compétitivité de l'accouplement des mâles et il est donc important d'examiner divers protocoles qui se sont révélés efficaces chez d'autres espèces d'insectes, comme le fractionnement de dose. Une dose entièrement stérilisante de 70 Gy a été administrée aux mâles *Aedes aegypti* en une dose unique ou fractionnée en deux doses égales de 35 Gy, ou une faible dose de 10 Gy suivie d'une seconde dose de 60 Gy. Les deux doses étaient séparées par des intervalles de 1 ou 2 jours. Des tests de longévité, d'aptitude au vol et de compétitivité à l'accouplement ont été réalisés pour identifier les effets bénéfiques des différents traitements. Des effets positifs de la dose de fractionnement ont été observés en termes de longévité des mâles et de compétitivité à l'accouplement. Bien que l'application de doses fractionnées améliore généralement les paramètres de qualité des mâles, les avantages peuvent ne pas compenser le travail supplémentaire dans les programmes TIS pour la gestion des moustiques vecteurs.

Introduction

Combatting mosquito species responsible for transmitting debilitating diseases to humans and animals has been a continuous challenge throughout history. Although undeniably, the development of insecticides and repellents was a major breakthrough and has been a powerful tool against mosquito vectors to date, many of the target species have evolved to develop insecticide resistance to most of the available chemicals [28, 30, 31, 38]. Furthermore, the extensive use of insecticides comes with detrimental adverse effects in people, animals,

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*Correspondence author: h.yamada@iaea.org

off-target and beneficial insects, and the environment [32]. The sterile insect technique (SIT) offers an alternative, "green", species-specific and sustainable tool for the management of insect pests and reduces the dependence on insecticide use [11].

The Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA) Insect Pest Control Laboratory in Seibersdorf, Austria is currently tailoring the SIT for its implementation against important human disease vectors, in particular *Aedes aegypti*, *Ae. albopictus* (major vectors of dengue, chikungunya, Zika, and numerous other arboviruses) and *Anopheles arabiensis*, an important vector of malaria. This includes the development of equipment, methods and guidelines for colonizing and mass rearing the target species, sex separation, sterilization by irradiation, handling, transport and release methods, executing field trials, and quality control (QC), of which the most notable advancements are reviewed in Vreysen et al. [36].

One of the challenges in the SIT for mosquitoes is balancing sterile male production efficiency with downstream sterile male quality. Increasing stress factors such as excessive handling, selective pressures of mass rearing, external stressors like irradiation exposure, chilling and packing are among the numerous sources of stress for the mosquitoes, and these can influence the overall male quality. A high level of biological quality in the sterile males is required for their success in the field once released. The factory-produced sterile males must outcompete their wild counterparts to mate with wild females. Only then will the target population decline with each successive generation [23, 24].

It is still unclear which stress factors are most important in reducing male quality, and what combinations of stress factors may further exacerbate this. Several factors known to cause a decline in male quality indicators have been investigated, such as the pressures of mass rearing [3], chilling and packing adults [6, 7, 42], hypoxic environments, for example, during irradiation procedures [39], irradiation exposure itself [18], and a combination of factors encountered during sterile male production [8, 34, 41]. Contrarily, some studies have shown that improving handling protocols can also improve male quality. Irradiation procedures including the preparation and handling methods, and the radiation exposure itself can decrease male quality if the males are overdosed, or if handling becomes excessive, and other stress factors such as chilling, and transportation are added [9]. On the other hand, improving irradiation protocols, such as performing the exposures in hypoxia or fractionating the total sterilizing dose into two or more smaller doses have been shown to greatly improve sterile male quality in various insect species: for example, dose fractionation improved longevity in boll weevils [21]; improved competitiveness was reported in the spotted bollworm after fractionated doses, whereas longevity and insemination capacity did not change. In the Indian meal moth, however, splitting the irradiation dose into three fractions improved longevity and mating propensity [5]. Fractionating a fully sterilizing dose in the West Indian sweet potato weevil maintained competitiveness for 12 days as opposed to just 6 days when given an acute dose [25]. Ducoff et al. [10] reported that the more the irradiation dose is fractionated, the better the survival in the confused flower beetle, and fractionating dose in the presence of nitrogen greatly improved tsetse fly longevity [35].

In this study, we investigated whether fractionating the irradiation dose needed to achieve > 99% sterility in *Ae. aegypti* (70 Gy in our setting), can improve male quality in *Aedes* mosquitoes. The total dose was split either into two equal units (35 + 35 Gy) or by "conditioning" the males with a low dose of 10 Gy, followed by the additional 60 Gy. A rest period of 1 or 2 days between exposures was also tested to see whether either would result in beneficial effects on longevity, flight ability, and mating competitiveness.

Materials and methods

Mosquito strains and rearing

A standard laboratory reference strain of *Ae. aegypti* [12, 14] was used for all experiments. The *Aedes* strain has been maintained following the "Guidelines for Routine Colony Maintenance of *Aedes* mosquito species" (FAO/IAEA, [12]).

Sample preparation

Pupae were collected and sexed based on pupal size dimorphism using a glass pupal sorter [16] and sex was verified under a stereomicroscope. Males were kept for treatment and females were placed in individual drosophila tubes for emergence to ensure virginity for later mating.

Adult males that emerged within a 12 h window were collected, batched in groups of 20, and kept in 15 × 15 × 15 cm Bugdorm® cages (MegaView Science Co. Ltd., Taichung 40762, Taiwan) until the following day when they were briefly knocked down in a cold room at 4 °C, transferred to, and irradiated in small 2 cL plastic cups closed with a sponge. At the time of the (first) irradiation, the adults were 24–36 h old.

Irradiation and dosimetry

Radiation treatments were performed in a Gammacell 220 (Nordion Ltd, Kanata, ON, Canada), which had a dose-rate of 59.1 Gy/min at the time of the experiment.

The dosimetry system used to verify the dose received by the samples was based on Gafchromic HD-MD-V3 film (Ashland Advanced Materials, Bridgewater NJ, USA) following the IAEA protocol [20]. Three films of MD film were packed in small (2 × 2 cm) paper envelopes and placed directly above and below the mosquito samples. Films were read with an optical density reader (DoseReader 4, RadGen, H-1118 Budapest, Sasadi út 36, Hungary) after 24 h of development.

A total dose of 70 Gy was applied for the experiments, expecting to achieve > 99% sterility, following previous irradiation dose-response experiments with this strain and irradiator [39]. Control groups were handled in the same way but were not irradiated (group A). Irradiation doses were applied to samples as follows: either an acute dose of 70 Gy (group B), or fractionated into 2 doses of 35 + 35 Gy, with either 1 day (group C) or 2 days (group D) of rest between exposures, and 10 + 60 Gy, with either 1 day (group E) or 2 days of rest (group F) between exposures (Table 1). Two biological

Table 1. Treatment groups, exposure intervals, and doses used (Gy).

Group	Interval duration	Irradiation dose(s)
A	Non-irradiated	0 Gy
B	Acute dose	70 Gy
C	1 day	35 + 35 Gy
D	2 days	35 + 35 Gy
E	1 day	10 + 60 Gy
F	2 days	10 + 60 Gy

repetitions with three technical repeats each were performed for each treatment and control group.

Assessing the dose response and male quality parameters following acute dose compared to fractionated doses with either a 1- or 2-day interval between exposures

Assessment of induced sterility

Following irradiation, the male adults were placed in 15 × 15 × 15 cm Bugdorm® cages with a supply of 10% sugar solution. Twenty virgin females were added to each cage and were allowed to mate for 3 days before they were provided with 2 bloodmeals on consecutive days (days 6 and 7 post-emergence). Oviposition cups containing water and germination papers were added to each cage on day 8 for *en masse* egg collection (on days 9 and 10 post-emergence), following routine rearing protocols [12]. Egg papers were collected, matured (slow-dried over 4 days) and stored for 10 days before hatching. The total number of hatched and un-hatched eggs were counted using a stereomicroscope. Any un-hatched eggs were either opened with a dissection needle, or if many, were bleached to determine the fertility status [13].

Assessment of longevity

Samples of 30 adult males were reared, prepared, irradiated and caged as described above. Dead individuals were counted and removed on weekdays until all were dead. Three repetitions were performed for each treatment group and controls.

Assessment of flight ability

Samples of 100 (±5) adult males were reared, prepared, irradiated and caged as described above. All samples were taken to the flight test device 1 day after the last irradiation exposure. (Note: As the flight test requires that all treatment groups and control are run at the same time, and with adults of the same age, sample groups B, C and E had 2 recovery days after the last irradiation exposure and prior to the flight test, whereas groups D and F only had 1 day of rest). The flight test was performed as described in [29]. Two biological repetitions with each two technical repeats were performed for each treatment group and control.

Assessment of mating competitiveness

To evaluate whether fractionating irradiation dose is beneficial in terms of resulting sterile male competitiveness, and whether 1 or 2 days of rest between exposures improves male quality, and whether 2 equal half doses (35 + 35 Gy) or a low dose followed by a high dose (10 + 60 Gy) results in more competitive males, two types of sterile males were offered to virgin females for direct competition as follows: B *vs.* C, B *vs.* E, C *vs.* D, and F *vs.* D. Samples were prepared as described in the Sample preparation and Irradiation and dosimetry sections. Males of the required groups were split into two groups. The males of one of the halved groups were fed with 0.4% rhodamine B (Sigma Aldrich, 95% dye content) in 10% sucrose solution, as described by Johnson et al. [22] to mark sperm, whereas the other half was not marked.

For each competitive mating cross, 10 marked males from one treatment group and 10 unmarked males from a second treatment group were transferred to a 60 × 60 × 60 cm cage (Bugdorm®). Ten virgin females were subsequently added to the cage and were left to mate for 3 h, as recommended by Li et al. [27]. Females from each mating cross were then removed and kept frozen for later dissection. A second cross was then set up using males from the same two treatment groups, but with reciprocal marking status. A competitive mating cross of marked and unmarked males that were not irradiated served as controls to assess whether the marking itself had an effect on competitiveness. Females were chilled and dissected under a steromicroscope and the spermathecae removed and viewed under a fluorescence stereomicroscope (Olympus BX41, Tokyo, Japan) using an RFP1 filter to determine insemination status and the presence/absence of Rhodamine B. Four biological repetitions were performed for each cross.

Statistical analysis

All statistical analyses were performed in R (version 4.1.0) using RStudio (RStudio, Inc. Boston, MA, USA, 2016). Generalized Linear Mixed Models (GLME, lme4 package) were used with the appropriate distribution family.

Male flight ability data were analyzed as response variable, treatment (6 levels: Treatment groups A–F) as fixed effect, and the repetition nested with technical repetition as a random effect considering each specific experiment.

Mixed Effects Cox Models ("coxme" function in "survival" package) fit by maximum likelihood with mosquito time to death as response variable, treatment (6 levels: Treatment groups A–F) as fixed effects, and repetition as a random effect, were used to analyze the survival of mosquitoes following the treatment in each specific experiment. Survival graphs were built using the packages "survival", "ggplot2", and "ggpubr". Multiple comparisons using the "emmeans" function (in package 'emmeans") were performed to observe differences between specific treatment groups.

For the competitiveness tests, the effect or marking was first analyzed to ensure there was no effect. Data were then analyzed per mating cross separately (2 levels: treatment 1 and treatment 2), regardless of marking status using binomial models.

The full models were checked for overdispersion using Bolker's function [4] (in package bblme). A p-value of less than 0.05 was used to indicate statistical significance in all cases.

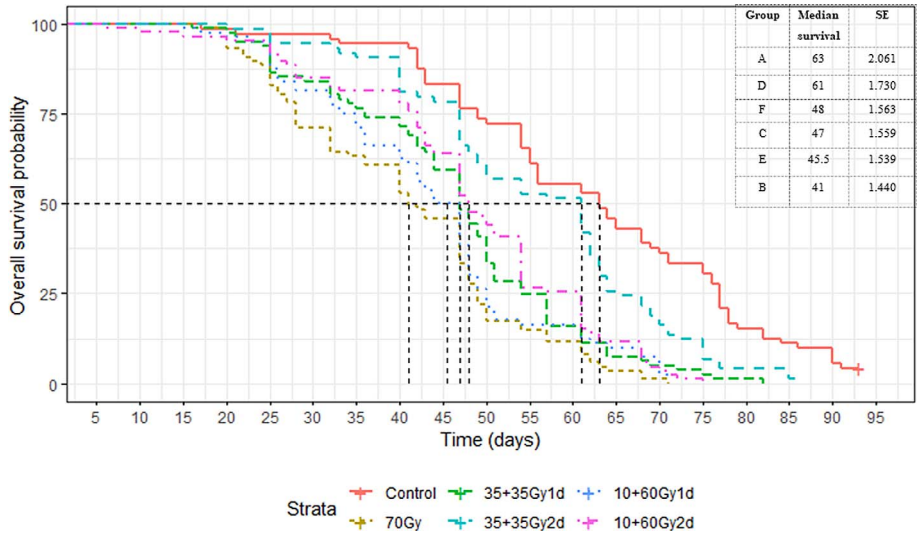

Figure 1. Survival curves of *Ae. aegypti* males sterilized with one acute dose or fractionated dose with 1- or 2-day intervals compared to untreated males. Table: Median survival (in days) of males in treatment groups A–F from highest to lowest.

Results

Dosimetry

The dosimetry confirmed that all doses received lay within a 3.07% error range (calibration MD film lot# 1222001; 2021.12.13).

Assessment of induced sterility

All irradiation treatments resulted in sterility levels beyond 99% in relation to non-irradiated controls (induced sterility). A dose of 70 Gy (group B) administered at once resulted in expected low levels of residual ferility of 0.007 ± 0.0026, whereas all fractionated doses (groups C–F with a total of 70 Gy) resulted in full sterility (100%), no matter the split dose proportions nor the number of days between exposures. There was a clear difference in induced sterilty after acute doses of 70 Gy and all fractionated exposures (χ^2 = 11.060, *df* = 3, $p < 0.0001$).

Assessment of longevity

Overall, non-irradiated control groups (A) lived longer than males in all other treatment groups (B–F) ($p < 0.001$), although group D was only slightly different from the Control ($p = 0.012$) (Fig. 1). Fractionation with a 1-day rest between exposures was not better than an acute 70 Gy dose, no matter how the dose was split (C *vs.* B: $p = 0.079$; E *vs.* B: $p = 0.682$), although the trend was still that the males from Group B (acute 70 Gy dose) performed the worst overall, especially after the first 3 weeks (Fig. 1). Fractionation with a 2-day rest between exposures was better than an acute dose, no matter how the dose was split (D *vs.* B: $p = 0.001$; F *vs.* B: $p = 0.025$). Two-day rest between exposures produced longer-lived males, no matter how the dose was split (D *vs.* C: $p = 0.0025$; D *vs.* E: $p = 0.001$). With a 2-day rest, the dose split into 35 + 35 Gy was more beneficial in terms of longevity than 10 + 60 Gy (D *vs.* F: $p = 0.009$; D *vs.* E: $p < 0.001$). The 1- or 2-day interval in the 10 + 60 Gy groups showed no difference in survival (F *vs.* E: $p = 0.586$). There was also no difference in the 1-day interval groups (C *vs.* E: $p = 0.843$). The full results of the multiple comparisons can be found in the Supplementary file.

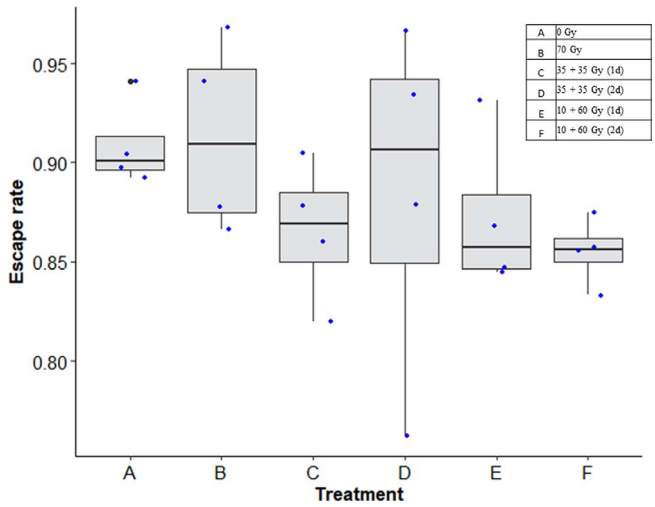

Figure 2. Escape rates of males irradiated with acute dose *vs.* fractionated dose with 1- or 2-day intervals, compared to non-irradiated control males.

Assessment of flight ability

Overall, the treatment had only a marginal effect on flight ability (χ^2 = 10.309, *df* = 5, $p = 0.0669$). However, treatment "F" (10 + 60 Gy, 2-day interval) had a lower escape rate (Fig. 2, $p = 0.0229$).

Assessment of mating competitiveness

When pooling data from 70 Gy acute dose treatments and all fractionated dose treatments, the competitiveness was higher in fractionned treatments ($z = -3.872$; $p = 0.0001$). Only the 35 + 35 Gy fractionation treatment showed better

Table 2. Competitivness index (C) of males sterilized by acute dose *vs*. fractionated dose, with 1- or 2-day intervals, and C of non-irradiated controls marked with Rhodamin B (Rhod+) or without marking (Rhod−).

Cross #	Treatment 1	Treatment 2	C (of Tx 1)	C (of Tx 2)	Estimate	*SE*	*z* value	Pr(>|*z*|)
1	70 Gy	35 + 35 (1)	0.304	**0.696**	−0.7732	0.349	−2.216	0.0267*
2	70 Gy	10 + 60 (1)	0.353	**0.647**	−0.5705	0.347	−1.644	0.1
3	35 + 35 (1)	35 + 35 (2)	0.369	**0.631**	−0.4964	0.339	−1.465	0.143
4	10 + 60 (2)	35 + 35 (2)	0.542	0.458	0.1625	0.3299	0.493	0.622
5	Control Rhod+	Control Rhod−	0.496	0.504	−0.05407	0.32892	−0.164	0.869

Values in bold represent a marked increase.* indicates statistical significance.

competitiveness than the single 70 Gy dose (Table 2, Cross 1). Males irradiated with a 2-day interval between exposures were equally competitive regardless of the way the dose was split (Table 2, Cross 4). The marking status had no impact on competitiveness (Table 2, Cross 5).

Discussion

This study was initiated with the aim of assessing the impact of radiation dose fractionation on *Aedes* male quality, as to date, no reports describing the effects of dose fractionation in mosquitoes in general are available. The fractionated dose of 70 Gy in two equal parts of 35 + 35 Gy was chosen following methods described in most historical studies on other insect species, and thus two equal medium doses seemed appropriate for this initial experiment. The second strategy of administering a low (10 Gy) dose, followed by a second higher (60 Gy) dose was based on the hypothesis that the initial low dose could serve as sort of "preconditioning", whereby the cellular repair mechanism is stimulated, and may protect against excess somatic damage in the second exposure. A dose of 10–15 Gy alone has been shown to improve longevity in mosquitoes due to radiation hormesis compared to unirradiated males [1, 15, 19, 40]. To avoid prolonging the male production duration in an SIT facility, no more than 2 fractionated doses were considered for this study. Nor were recovery periods of more than 2 days considered between exposures, as it has been recommended to release the sterile males at around day 4 or 5 at the peak of their flight and mating activity, after which the flight ability begins to decline [29]. One and 2 days were selected as intervals also to ensure that there was sufficient time for the males to recover not only from the effects of the first irradiation, but also from the stress of handling before and during exposures, as it has been shown that, for example, flight ability is restored when males are given 1–2 days of rest post-exposure [29]. Selecting the length of intervals beween exposures is important and the ideal timing is not known for this species. The various publications describing dose fractionation studies in insects all have different intervals and number of exposures. A 4-hour interval between radiation doses allowed for some tissue recovery in the cotton leaf worm, whereas 2 h did not [37]. Increasing interval duration in tsetse flies from 1–2 days to 5 days also allowed recovery of chromosome damage and thus resulted in higher fertility rates in irradiated males [35]. Two doses with either 1 day, or 2 day intervals, or 3 doses were administered to West Indian sweet potato weevils (*Euscepes postfasciatus*) where it was found that fractionating the irradiation dose prolonged mating propensity significantly [25]. Other studies selected other intervals: 3 doses over 1–3 days for the Indian meal worm *Plodia interpunctella*, [5], 2, 3, or 4 equal doses with 2 h intervals in the spotted bollworm *Earias vitella*, [33], and 5 fractions with intervals of 1 min, 10 min, 1 h and 1 day in the grain beetle *Calandra granaria* [21]. Why these interval durations or number of fractions were selected was not clearly explained in most of the articles.

In our study, the acute sterilizing dose of 70 Gy achieved the expected sterility level of > 99%, with a few eggs hatching only, whereas the same dose fractionated resulted in 100% sterility with no eggs hatching in any of the batch samples, in all repetitions. This was unexpected as most other studies on dose fractionation in insects found that splitting doses resulted in less sterility than the equivalent acute dose [1, 5, 21]. However, Vreysen and Van der Vloedt [35] found that fertility increased when the interval durations increased, but was still less than that of males irradiated with an acute dose. Shantaram et al. [33] reported that sterility induced in the spotted bollworm (*Earias vittella*) was the same in males irradiated with an acute or fractionated dose, whereas other lepidopteran species presented reduced sterility levels following dose fractionation. A possible explanation is that male spotted bollworms emerge with a full set of sperm and there is no further multiplication of spermatogonia. One hypothesis is that sterility levels in some insects are significantly influenced by the timing of radiation exposures, depending on the process and timing of spermatogenesis occurring. If spermatids are fully formed, the effects of irradiation in either one acute dose, or several fractionated doses may not affect the final sterility level. In mature sperm of *Drosophila*, there was no effect of exposure to acute or chronic doses while in spermatids, increased genetic damage was observed when the dose was split [2], and thus increased sterility, as was observed in this study. The authors of the study proposed that oxygen was somehow released in the cellular components between the radiation doses, and thus increases radiation damage during the second dose. The observation that there was less biological damage with dose fractionation in argon than when oxygen is present supports this hypothesis. This notion is supported by Haynes et al [17] who suggested that fractionation or lowering dose rates may allow regeneration of sub-lethal cell damage, but increasing the number of fractions will reverse the beneficial effects; i.e., repeated radiation doses cause cells that were radioresistant due to hypoxia during previous doses to reoxygenate, and thus become 2–3 times more radiosensitive in subsequent exposures. Another possibility is that the chromosome breakage and/or repair mechanisms are affected, and this in turn depends on the stage of spermatogenesis. In sperm reaching

maturity, a higher (subsequent) dose may be needed to reach the target sterility. In any case, it seems that spermatids and spermatozoa have different radiotolerance [35]. In a study in mice, Leonard and Deknudt [26] separated two fractionated doses by increasing time intervals. They concluded that the translocations caused by the second exposure were not all affected by or related to the damage caused by the first exposure, and that the fractionated interval effect was more related to the cell cycle; i.e., the second dose was either received by a radiosensitive or radioresistant stage of the cell cycle.

Although the historical publications reviewed in this study have reported differing effects of fractionation intervals on sterility levels and suggest different hypotheses on why this is the case, most studies agree that dose fractionation improved one or more male biological quality parameters. Few have reported no or negative effects. However, it is important to note that the number of fractions and time intervals are important for the outcome and thus changing these variables may have resulted in a better outcome in the particular insect studied. In our study, splitting the sterilizing dose for *Ae. aegypti* males into two fractions, with an interval of 1 or 2 days, improved longevity in all treatment groups as compared to the males irradiated with one acute dose. The trend showed that males receiving 2 days rest between doses survived longer than those with only 1-day rest. In both the 2-day interval groups and the 1-day interval groups, the males exposed to 2 equal doses of 35 Gy survived longer than those irradiated with a low dose (10 Gy) followed by a high dose (60 Gy). This may be because 60 Gy is still a relatively high dose, and not much reduced from the total acute dose of 70 Gy.

There was no difference observed in flight ability between males subjected to acute or fractioned doses. All treatment groups performed equally as compared to non-irradiated control groups, except treatment group F. This result suggests that subjection to one high dose, *or* the double handling, *or* only having one recovery day is tolerable in terms of flight ability; however, when all three factors are combined, this reduces the overall male quality, which is reflected by the reduced escape rates [29]. Although not statistically significant, the trend was that the double handled males all had the lowest recorded escape rates (C–F), when compared to the low scores of the males handled only once (A and B), suggesting that stress from handling can be more detrimental than irradiation itself [9].

Overall, there was no observed difference between males receiving two equal medium doses, or one low then one high, except for males exposed to two doses of 35 Gy, which showed better competitiveness. A 2-day interval provided better recovery than a 1-day interval both in the longevity and flight ability tests.

Conclusions

Different insect species may be more susceptible to acute doses of irradiation, and these may benefit from fractionation. Others may be more sensitive to increased handling and stress. Handling of adult mosquitoes in preparation for irradiation includes briefly chilling the adults and aliquoting batches into separate tubes, (or compacting large numbers of chilled adults for mass irradiation), transportation to and from the irradiation facility and then back to the insectary. Considering that males subjected to fractionated doses had double handling and still performed better in the survival assays and maintained this trend in competitiveness tests showed that dose fractionation does seem to reduce overall radiation damage in this species. However, the question still remains whether the biological benefits of dose fractionation outweigh the additional labor and thus reduced production efficiency in mosquito SIT programmes. It would be essential to assess the competitiveness of the sterile males resulting from the various fractionation treatments in the field, and the duration of any improved competitiveness over several days as was done, for instance, for the West Indian sweet potato weevil [25]. Other combinations of split doses and recovery periods may result in a better outcome and may warrant the extra efforts. The marginal improvements in longevity and mating competitiveness in the laboratory suggest that dose fractionation into two equal doses may only be recommended for this mosquito species if these quality improvements are confirmed in the field.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HY conceptualized the experimental designs for the experiments, carried out the experiments and drafted the original manuscript. HM carried out the flight tests and contributed significantly to the data analysis and later versions of the manuscript. CK, WM, NSBS and TW provided all live material following standardized rearing procedures and assisted in the experiment set-up and data collection. JB and HM contributed to the experimental designs and carried out the statistical analyses. JB supervised and supported the project. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study, including all dosimetry reports, are available from the corresponding author upon reasonable request.

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Supplementary materials

The Supplementary materials of this article are available at https://www.parasite-journal.org/10.1051/parasite/2023005/olm

Figure S1. Multiple comparisons of means: Tukey contrasts.

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Characterization and dose-mapping of an X-ray blood irradiator to assess application potential for the sterile insect technique (SIT)

Yeudiel Gómez-Simuta [1], Andrew Parker [2], Carlos Cáceres, Marc J.B. Vreysen, Hanano Yamada [*]

Joint FAO / IAEA Centre of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna International Centre, PO Box 100, 1400 Vienna, Austria

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ABSTRACT

Self-contained gamma irradiators have been extensively used to reproductively sterilize insects for the sterile insect technique (SIT). More recently, the use of X-ray generators has gained attention due to the reduced investment, logistic, regulatory and safety requirements involved in the procurement, transport and operation of these machines compared with gamma irradiators. In this study, we evaluated a commercially available, "off-the-shelf" X-ray blood irradiator and found it suitable for insect irradiation in the frame of the SIT.

1. Introduction

The sterile insect technique (SIT) (Dyck et al., 2021) as a part of area-wide integrated pest management strategies (Klassen and Vreysen, 2021) has mostly relied on the use of gamma radiation from isotopic sources (Bakri et al., 2021), and remains to date the most efficient and reliable method to sterilize insects before their release in the field, especially in larger programmes. However, the already high and still rising costs of radioactive sources such as cobalt 60 (^{60}Co) makes this a limiting factor for many SITprojects, especially in the initial phases of the project when funding might be limited. Not only the radioactive source itself is costly, but the transport and required infrastructure to house the irradiators require high initial investments. Furthermore, procurement of radioactive sources is becoming increasingly difficult and regulations with respect to its purchase, transport and use, more stringent. Accordingly, the use of X-ray irradiation has attracted great interest in recent years due to their lower purchase cost, ease of transportation, and comparatively simpler regulatory requirements during the transport, import and operation. The Insect Pest Control Laboratory of the Joint FAO/IAEA Center of Nuclear Techniques in Food and Agriculture has been exploring the suitablilty of electron beams (e-beam) or X-ray technologies for the application of the SIT against insect pests (IAEA, 2012). At the moment accelerator systems for generating electron beams for direct use or for conversion to X-rays are too large, complex and expensive for SIT programmes, although compact accelerators are under development. Interest, therefore, centres around systems operating in the 150–225 kV range with orthovoltage tubes.

Gamma photons from ^{60}Co (average energy 1.24 MeV) have a higher energy than orthovoltage X-ray photons (150–225 keVp, the peak photon energy in the X-ray beam), but the chemical and biological effects are similar, as both photons cause indirect ionization leading to the production of ions and free radicals in the irradiated materials (Cleland and Stichelbaut, 2013). Although e-beam and X-ray technologies employ ionizing radiation, they are not produced by nuclear processes, thus these technologies may be more acceptable to the public because they produce no radiation when switched off, and there are no transportation or radioactive waste issues (Bakri et al., 2021). Ionizing radiation consists of charged particles such as electrons (directly ionizing radiation) or uncharged particles such as photons (indirectly ionizing radiation) that can generate free high-energy electrons in the material being irradiated by the Compton or photoelectric effects; these high energy electrons then cause further ionization (secondary electrons) and are the principal cause of the observed radiation effects. The nature of the energy transfer from radiation (photons or electrons) to the irradiated matter influences the rate of absorbtion (attenuation) of the radiation beam and hence the distribution of dose in the target material; electrons interact strongly with material so the beam is absorbed in the

* Corresponding author.
E-mail addresses: yeudielgomez@hotmail.com (Y. Gómez-Simuta), a.g.parker@protonmail.com (A. Parker), C.E.Caceres-Barrios@iaea.org (C. Cáceres), M.Vreysen@iaea.org (M.J.B. Vreysen), h.yamada@iaea.org (H. Yamada).
[1] Programa Operativo Moscas. IICA-SENASICA. Km 19.5, Carretera Tapachula-Ciudad Hidaldo, Metapa de Dominguez Chiapas, Mexico. C.P. 30860
[2] Roppersbergweg 15, 2381 Laab im Walde, Austria.

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material within a short distance but photons interact more weakly and so penetrate further into the material, generating secondary electrons deeper within the body of the material (IAEA, 2002). The dose variation in the irradiated matter (sample) is an important parameter for insect irradiation (Bakri et al., 2021; Mehta, 2017). Because the design of an irradiator can affect the dose distribution and the attainable dose range, the dose distribution within the sample canister must be determined (Miller, 2005) and this can be done by dose mapping.

The radiation dose that is absorbed by the treated insects to obtain a particular level of sterility is species specific and therefore of critical importance to SIT programs (FAO/IAEA, 2003). Many insects can be irradiated at doses less than 120 Gy to achieve high levels of reproductive sterility (Bakri and Hendrichs, 2002) but some groups are more radioresistant and require higher doses for full reproductive sterility, such as Drosophilidae (120–200 Gy) (Lanouette et al., 2017; Nikolouli et al., 2017; Sassù et al., 2020) and Lepidoptera (more than 200 Gy) (Chakroun et al., 2017; Vreysen et al., 2016). In any case, the dose to be used for any given SIT program should be based on mutual agreement between the rearing facility and the end user and the decision is taken based on the results of the sterility test, i.e. biological dosimetry, in combination with considerations such as program requirements and effects of radiation dose on insect quality (FAO/IAEA, 2003; Parker and Mehta, 2007; Parker et al., 2021).

Considering that the determination of accurate dose response curves for the target insect, efficiency of processing capacity, and reliability of the irradiator regarding dose given and its uniformity within the sample are prerequisites for any programme releasing sterile insects, it is important to characterize irradiation devices before their use in SIT programs. In this study, we describe the dose mapping of an X-ray blood irradiator (Raycell Mk2, Best Theratronics Inc., Kanata, Ontario, Canada) to evaluate its application potential for insect sterilization in the frame of the SIT.

2. Materials and methods

2.1. Characterization of the Raycell Mk2 blood irradiator

The irradiator consists of a cabinet containing two X-ray tubes, a control system, two power supplies, a radiation shielding chamber, control electronics, two removable shielding access panels to the irradiation chamber, operator controls, and a separate heat exchanger cooling system. The irradiator is supplied with a 2 L sample canister (167 diameter x 97 mm); a 3.5 L version is also available. The dose is delivered by the two opposing ortho-voltage tubes running at 160 kV. The sample canister is located centrally between the two X-rays tubes (Fig. 1a). The dose is controlled by setting the irradiation time, based on the central dose rate.

2.2. Dosimetry system

2.2.1. Dose rate at reference position

A Farmer type 0.18-cm^3 free air ionization chamber (10X6-0.18, RadCal Corporation, Monrovia, CA. USA) in conjunction with a digitizer and electrometer (AccuDose Model 9660A) was used as a reference dosimetry system to measure the dose rate and accumulated dose at a designated reference position. The ion chamber system was calibrated by the John Perry Laboratory (St George's University Hospital Trust, London) with traceability to the National Physical Laboratory with a calibration factor of 1.0 and uncertainty of 3.3% (k = 2) in the energy range 40–1250 keV. Dose rate measurements were taken by filling the canister with instant rice to simulate the radiation conditions normally used for insect treatment; instant rice was used as phantom material, as the density of Tephritid pupae and instant rice are quite similar, 0.46 and 0.44 g cm^{-3}, respectively (Mehta and Parker, 2011). The dose rate was determined at the center of the canister under the manufacturer's specified operational conditions at 160 kV. The measured dose rate at the reference position was used to calculate the time needed to obtain 100 Gy as the nominal dose. In order to determine the maximum and minimum dose throughout the irradiation canister, the canister was divided into three levels (Fig. 1b), identified as top, center and bottom. Five points at each level (A, B, C, D and E) were measured using the same ion chamber with the electrometer in cumulative dose mode, filling the canister with instant rice to keep the ion chamber fixed in each position during the irradiation process. By design, the sample canister is placed with the interlock system of the top of the canister in the direction of a safety switch located at the cathode side of the X-ray beam clearly labelled in the irradiation chamber (Fig. 1b). The anode side of the chamber is also labelled and clearly indicated on the opposite side of the

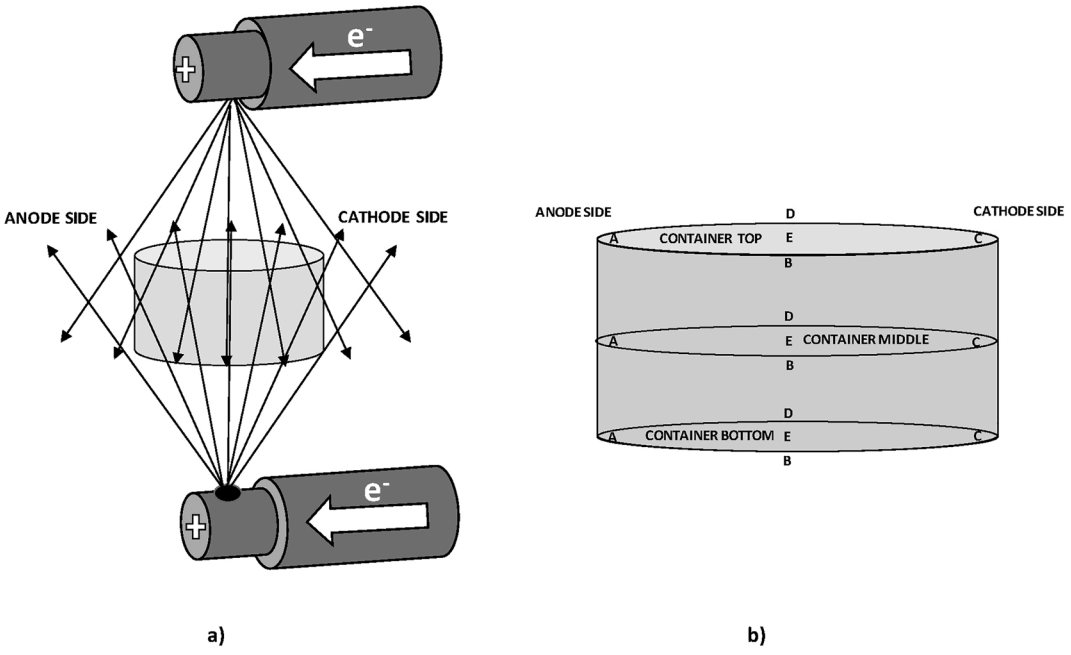

Fig. 1. Diagram of X-rays configuration: (a) The position of the irradiation canister at the middle of the two opposing X-rays tubes: (b) Distribution of the 15-reference point selected to measure the dose rate within the irradiation canister of the Raycell Mk2 X-ray blood irradiator.

canister interlock. These reference points were used to locate the position of the reading points: A (anode side), B (at the right side of the anode), C (cathode side), D (left side of the anode) and E (center of the respective level). Dose rate readings were performed 5 times for each position.

2.2.2. *Scanning Gafchromic™ dosimetry film*

The dosimetry was carried out using Gafchromic™ HD-V2 film (Ashland Advanced Materials, 1005 US Hwy No 202/206, Bridgewater NJ 08807, USA) that have a dose response range appropriate for insect irradiation and can be used in both X-ray and gamma irradiators. At low voltage X-ray energies (150 keV) the acetate backing sheet provides sufficient buildup material on one side but buildup material has to be supplied on the side with the active layer exposed (Ashland, 2021). Radiochromic films can be used to measure doses over the entire area of the sheet used and have a good resolution in the order of tens of micrometres. Whilst individual 10 × 10 mm dosimeters can be read with a densitometer, much finer resolution was achieved by scanning the film on a 48 bit per pixel professional grade flat-bed colour scanner (Epson Expression 12000 XL, Seiko Epson Corp. Japan) and using the colour channel information following the procedure of FAO/IAEA (2020) using tsplit and Microsoft® Excel® 2016. Gafchromic film, however, sometimes exhibits small imperfections of 1 mm or less that distort the dose readings. In order to more accurately represent the true dose distribution, the mean dose and dose range were calculated both over the total film area and after excluding the highest and lowest 1% of readings.

2.2.2.1. Calibration. The calibration of the film system was done using pieces of the film cut into 20 × 20 mm squares. A series of films were exposed to doses of 50, 75, 100, 125 and 150 Gy, each dosimeter placed separately in an envelope (FWT80, Far West Technologies, Goleta, California) and irradiated at the center of the canister filled with instant rice at the reference position. The exposure time was calculated according to the dose rate given by the reference dosimetry (described in section 2.2.1). The calibration doses were selected with two doses above and two doses below the nominal dose of 100 Gy. A zero-dose (non-exposed) film piece was used as control. The range of 50–150 Gy was selected for the calibration to allow dose uniformity ratio (DUR) values up to 3 to be measured without extrapolation as DUR values greater than 1.5 are unsuitable for the SIT. The reference position selected for the calibration was the center of the canister and was surrounded by instant rice to simulate the radiation conditions normally used for irradiating fruit fly pupae. The overall uncertainty in the dose measurement was estimated by summing in quadrature the uncertainty from the ion chamber calibration, the residual deveations in the regression fit and the coefficient of variation of individual pixel values in the 100 Gy calibration piece reading area, using a spread factor of 2 (ISO/ASTM, 2011).

2.2.2.2. Dose mapping. A central dose of 100 Gy for the mapping was selected. The purpose of mapping was to get the relative dose at different points to calculate the DUR. As was done for the calibration process, Gafchromic™ HD-V2 film was used. As the dose is delivered by two opposing X-ray tubes with the sample canister located centrally between them, two-dimensional dose distributions within the 2.0 L canister were measured using two different Gafchromic sheets inside the canister filled with instant rice.

In order to cover the vertical gradient from the top to the bottom of the irradiation chamber, a 16.5 × 8.5 cm rectangular Gafchromic™ film sheet ("V"), was placed inside the canister (Fig. 2a). The film was held between two 2-mm cardboard sheets, placed vertically at the center of the canister filled with instant rice and irradiated at a nominal central dose of 100 Gy.

To analyse the area located at the midpoint of the two opposing X-ray tubes, a second circular sheet of Gafchromic film 16.3 cm diameter was used, placed horizontally ("H") in the canister (Fig. 2b). The film was held between two 2-mm cardboard sheets, placed in the middle of the canister filled with instant rice and irradiated at a nominal dose of 100 Gy.

The first "V" sheet was removed from the cardboard and placed on the scanner 24 h after irradiation together with the calibration pieces and scanned at 75 dpi. The tiff files obtained were processed to separate the colour channels and adjacent blocks of 6 × 6 cells were summed to give a final resolution of approximately 2 × 2 mm. The channel values were saved separately as comma separated value (csv) files for further processing in Microsoft® Excel® 2016. After trimming to remove the values outside the extent of the films and edge effects (from cutting the

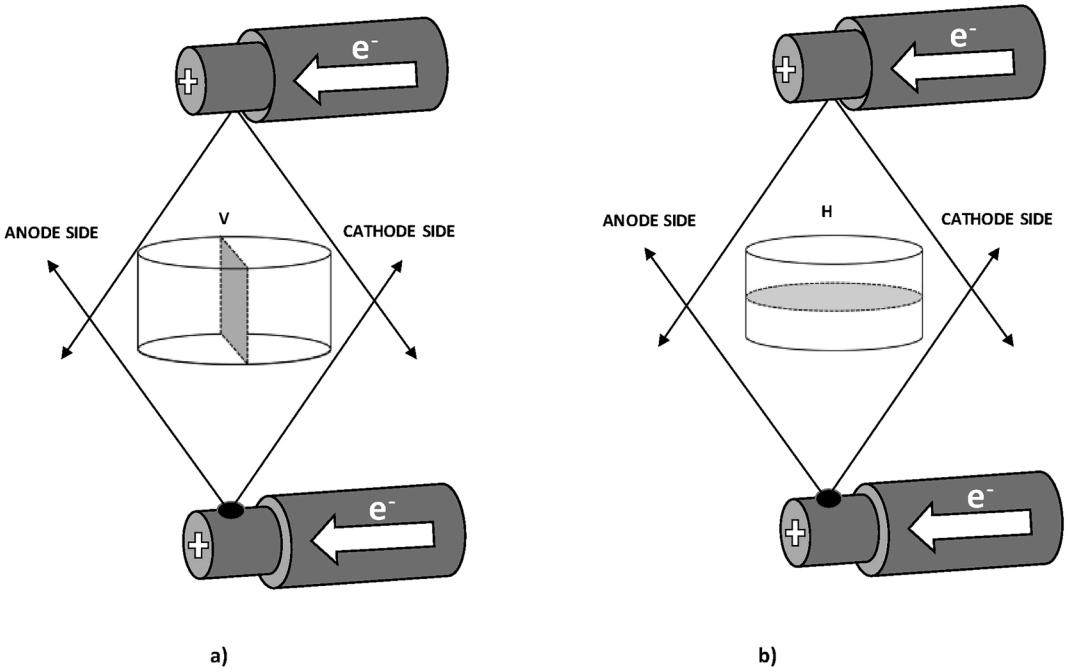

Fig. 2. Diagram of the position of the Gafchromic™ film inside the canister for mapping dose. (a) "V" Vertical Gafchromic film and ("H") Horizontal Gafchromic circular sheet.

film and from the binning process, which at the edge of the film combines values from the film with values from the background), for each colour channel the average was taken of the 5 × 6 block of cells in the middle of each calibration film and transformed to reciprocal as 2 × 10^6/x for each value for analysis. These values were plotted against the applied doses and a linear regression function calculated. The colour channel giving the highest R^2 value for the linear regression was selected and the relationship used to transform the dose map values to dose in Gy and then to dose relative to the average dose. The data in the relative dose maps were selected to create a surface plot. Additional details of this procedure are presented in FAO/IAEA (2020).

After irradiation, the second circular Gafchromic sheet ("H"), was removed from the cardboard and placed on the scanner together with the calibration pieces to be scanned and analysed under the same procedure described for the rectangular sheet.

The ion chamber dosimetry results are presented as dose ±1 sd.

3. Results

3.1. Dose rate of the Mk2 Raycell at the reference position

The average dose rate of the 15 reference points was 7.7 Gy/min. The Radcal ionization chamber registered a maximum dose rate of 8.2 Gy/min at the center top of the canister and a minimum dose rate of 7.2 Gy/min at the anode side of the bottom of the canister (Table 1). Based on the data of the minimum dose rate, the timer was set at 14.0 min to achieve the target dose of 100 Gy as minimum dose within the canister. The 15 points measured inside the canister registered an average dose of 107.3 ± 4.1 Gy. The lowest dose (100.6 Gy) was recorded at point A on the anode side of the bottom of the canister and the greatest dose (115.0 Gy) at the center top of the canister. The DUR calculated was 1.14 for the whole canister.

3.2. Calibration of the Gafchromic™ scans

Following the FAO/IAEA (2020) procedure, the calibration data were transferred to Excel files and the green colour channel was used for the analysis. The scatter graph of applied dose against 2 × 10^6/x and the linear trend line with the equation and R^2 value was obtained (Fig. 3). The R^2 value of the plotted graph was 0.997, indicating a good fit and the overall uncertainty in the dose measurement was ±6.4% (95% confidence interval, k = 2) composed of the uncertainty from the ion chamber calibration (3.3%), the uncertainty from the calibration regression line residuals (5.2%) and the uncertainty from the film response variability (1.4%) added in quadrature.

The values of the intersect (−156.07) and gradient (116.79) of the regression were used to transform the value of each cell of the dose maps to dose in Gy.

3.3. Dose mapping

The average dose for the whole "V" vertical position film was 96.9 ± 4.3 Gy. The dose variation from the top to the bottom of the irradiation chamber was calculated relative to the 96.9 Gy average dose, using the data in Gy of each cell (calculated dose/96.9 Gy). The vertical relative dose mapping values from the scanned Gafchromic film are shown in Fig. 4. The overall DUR was 1.31 or 1.21 after excluding the highest and lowest 1% of readings. The dose distribution was slightly asymmetric in the vertical axis, indicating that the upper X-ray tube is generating a higher output or the sample canister is not exactly in the middle between the two X-ray tubes.

Using the same procedures, the average dose for the whole horizontally positioned circular sheet placed in the middle of the canister ("H") was 93.3 ± 4.0 Gy. However, Fig. 5 shows a rapid drop in indicated dose at the bottom of the figure and examination of the data indicates an inconsistency in the performance of the Gafchromic film in three 2 mm strips of the film, representing a total of 57 data points (out of 4947). As we could not determine any mechanism for such an abrupt drop, we concluded that the film was faulty and excluded these 57 data points from further analysis. With these points excluded the average dose was 93.5 ± 3.6 Gy. The dose variation around the middle area of the irradiation chamber was calculated similarly relative to a 93.5 Gy average dose. The horizontal relative dose mapping values from the scanned Gafchromic film are shown in Fig. 5. The overall DUR was 1.28 or 1.19 after excluding the highest and lowest 1% of readings. The dose at the reference position and the dose map indicated that the minimum dose was received at points located at the anode side of the canister clearly labelled in the chamber (Fig. 1, Table 1). This is expected due to the anode heel effect, which refers to the lower field intensity towards the anode compared to the cathode side due to the lower x-ray emissions from the target material at angle perpendicular to the electron beam. In other words, photons emitted towards the X-ray tube's anode side are attenuated more than those emitted towards the cathode (Fuchs, 1947).

Combining the dose variation vertically and horizontally gives an overall DUR of 1.32 or 1.21 after excluding the highest and lowest 1% of readings.

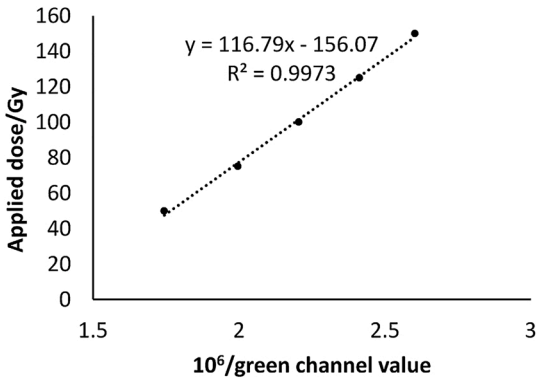

Fig. 3. Calibration curve for the Gafchromic film showing the trend line and equation obtained from doses of 50, 75, 100, 125 and 150 Gy irradiated in the Raycell Mk2 X-ray blood irradiator.

Table 1
Dose rate at three different levels within the canister (in rice): A (at the anode side), B (at the right side of the anode), C (at the cathode side), D (at the left side of the anode and E (at the center of the respective level); the average was 7.7 ± 0.3 Gy min^{-1}, the minimum dose rate was located at the anode side of the bottom of the canister.

Level	Position				
	A	B	C	D	E
Top	7.48	7.63	7.87	7.85	8.21
Center	7.30	7.58	7.63	7.53	8.10
Bottom	7.19	7.70	7.55	7.39	8.01

4. Discussion

The SIT relies on the release of insects that are both competitive with the wild insects and have a high level of reproductive sterility. Gamma and X-rays are used to sterilize the male insects, but they can also compromise the competitiveness of the released male insects as they damage tissues other than the gonads. The requirement for sterility places a lower bound on the dose that can be used whilst the requirement for competitiveness defines a maximum acceptable dose and hence defines the maximum acceptable DUR. Thus a balance has to be maintained between sterility and competitiveness (Parker and Mehta, 2007) and the closer the applied dose can be controlled the easier this is.

X-rays from conventional isovoltage tubes are produced in a cone

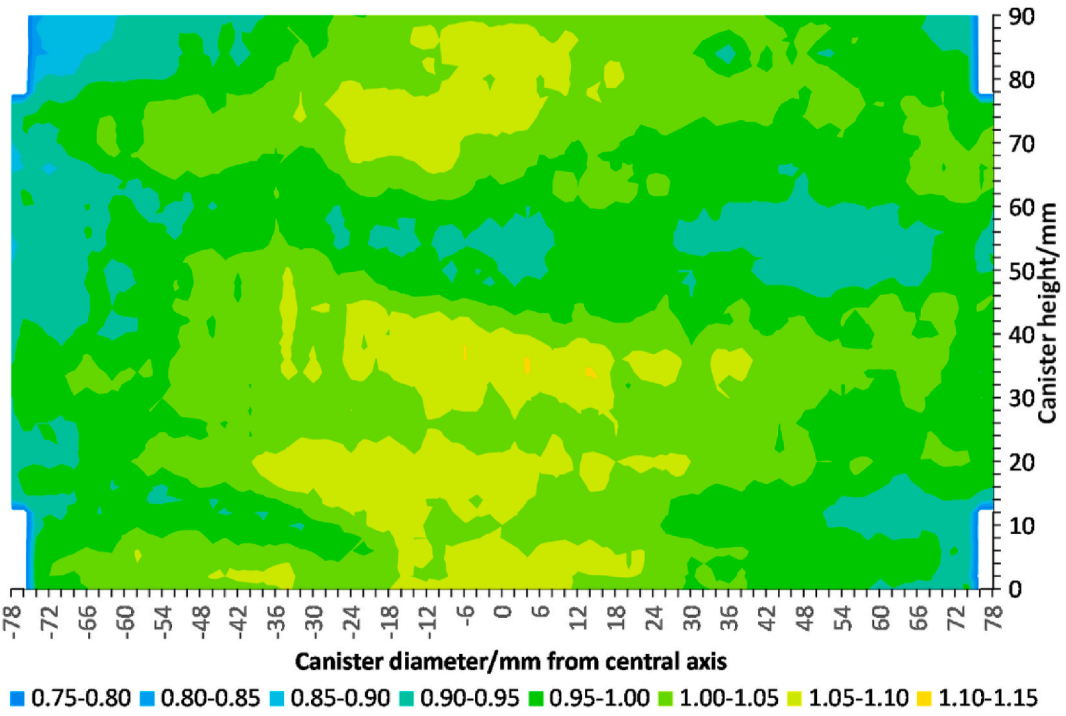

Fig. 4. Vertical dose map within the 2 L canister of the Raycell Mk2 X-ray irradiator showing the vertical dose distribution relative to the mean dose through the canister perpendicular to the anode-cathode axis. The blue edge at the corners is an artefact of the Excel surface plot routine.

from the anode so the dose rate falls as the square of the distance from the anode. In addition, the X-ray beam is attenuated by the material through which it is passing with the attenuation depending on the photon energy. At an energy of 160 keVp an X-ray beam is attenuated to half its original intensity by about 23 mm of water. These two effects mean that the dose delivered varies continuously within any sample. This variation can be reduced by rotating the sample on an axis perpendicular to the central axis of the beam or, as in the case of the Raycell Mk2, by having opposing X-ray beams resulting in a much smaller DUR that seen in single beam machines without rotation.

As it is desirable that all insects in a given canister receive nearly the same dose, the identification of areas with minimum and maximum dose is very important for the SIT (Bakri et al., 2021). Dose mapping is a useful tool to determine the pattern of the dose distribution in an irradiation chamber. Our findings on the characteristics of the Raycell Mk2 X-ray irradiator show that the vertical dose variation inside the irradiation canister was very small, with a DUR of 1.14 from the measurements with the ion chamber and with most of the measurements lying close to the average dose of 107.3 ± 4.09 Gy. The manufacturers quoted DUR, based on blood bags, is 1.5 (Best Theratronics Ltd, 2021). The much lower DUR found here cannot be explained fully by the difference in density of the loads used for the measurements (0.44 g cm^{-3} against c. 1 g cm^{-3}) and suggests that the manufacturer's figure may be overly pessimistic. It is likely that the DUR in the 3.5 L alternative canister may be acceptable (<1.3). The DUR compares favourably with the DUR of 1.3 for another X-ray irradiator (the RS2400, early model) (Mehta and Parker, 2011).

The minimum dose of 100.6 Gy at the anode side of the bottom of the canister can be used as a reference point for routine dosimetry, giving assurance that the desired absorbed dose will be achieved. This data helps to ensure that all insects in the irradiation canister will receive the dose in the specified range, fulfilling one of three main process control elements recommended for the SIT to avoid release of insects that are significantly under-dosed. The three quality control elements which support various steps in the irradiation process are: sterility testing, routine dosimetry and radiation-sensitive indicators and thus complement each other (FAO/IAEA/USDA, 2019; Parker and Mehta, 2007). Considering the DUR value of 1.14 within the canister, the excess dose delivered in the other parts of the irradiation canister are not likely to cause detrimental effects on the biological quality of the resulting sterile insect, showing the advantage of the position of the irradiation chamber (and canister) between the two opposing X-ray tubes. The minimum cumulative dose of 100.6 Gy obtained during the esposure time of 14 min gives a dose rate of 7.1 Gy/min for the Raycell Mk2 blood irradiator for SIT purposes.

The resolution of the mapping possible with the ion chamber is limited by its physical dimensions. The chamber itself is about 10 mm diameter on a stem of 12 mm (RadCal Corporation, 2021). The center of the sensitive volume is about 9 mm from the tip, so the closest the center can be to the edge of the canister is 6–9 mm. Gafchromic™ film offers much higher resolution, to about 10 μm. Whilst such high resolution is not useful, a resolution of 1–2 mm can be used to characterize the field in the canister to the edge of the useable volume, capturing variations not shown by the point measurements with an ion chamber. As the film measures everywhere it will capture a higher dose range than the ion chamber, in this case 1.32 or 1.21 when the highest and lowest 1% of measurements are excluded. The value of 1.21 is somewhat higher than that derived from the ion chamber measurements, but still indicates that this irradiator will be suitable for SIT use.

Ionizing radiation produced by electric energy has the advantage that the delivered dose is not affected by decay factors found with radioactive sources. The gradual decay of the radioactive sources decreases dose rates over time, thus changing exposure times and conditions, and eventually requires replacement of the source. This again involves specialized and costly procurement, transport and installation. However, X-ray irradiators also come with running costs, and need servicing as well as periodic replacement of the X-ray tubes, and reliable power supplies. Under operational conditions, the Raycell Mk2 could irradiate 13.4 million fruitfly pupae per week with one 6 h shift per day, with the potential to increase the processing capacity to 26.9 million per week with the implementation of a second shift per day. Approximately 100,000 *Aedes* adults can be irradiated in a 1L canister if immobilized

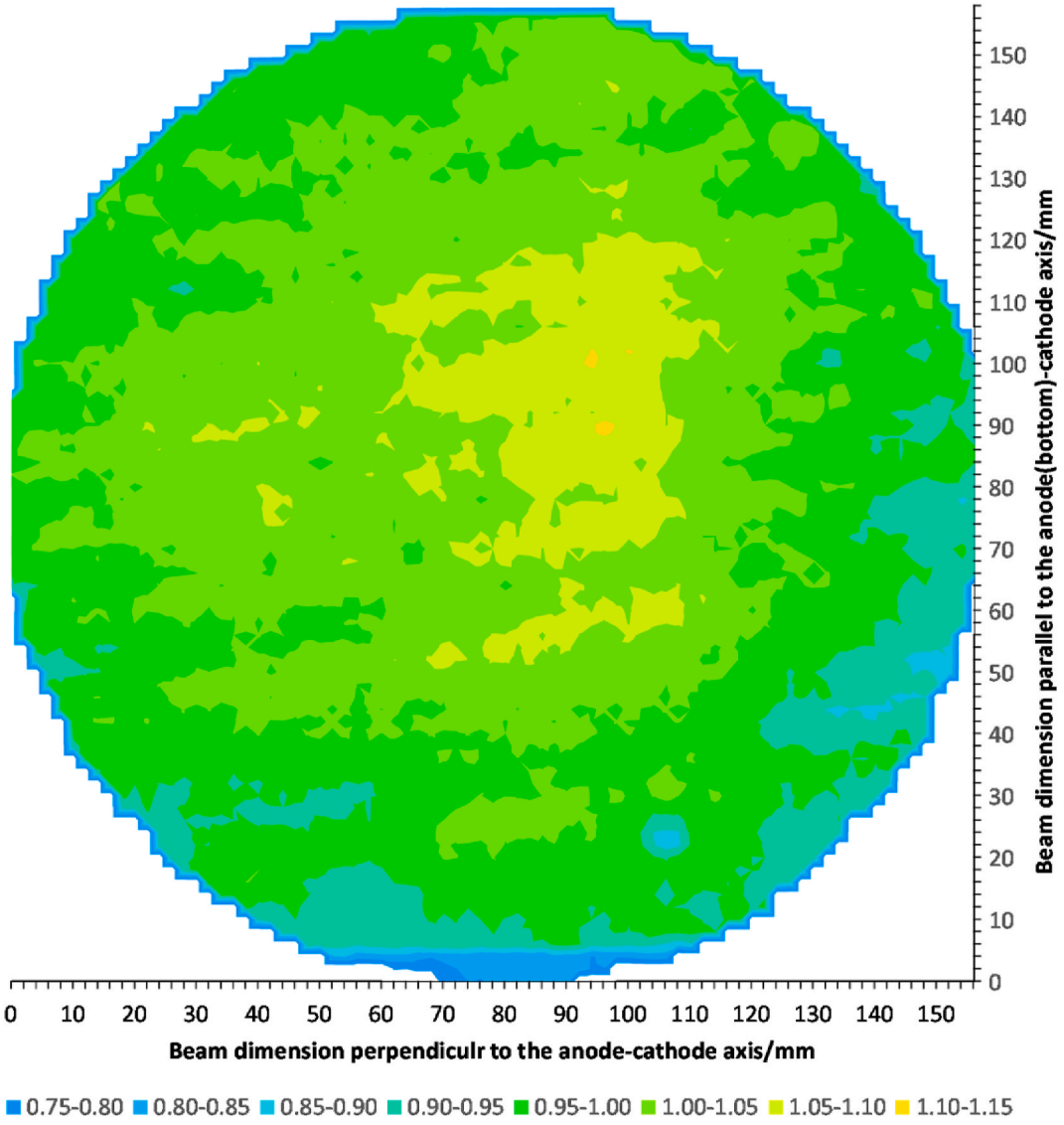

Fig. 5. Horizontal dose map within the 2 L canister of the Raycell Mk2 X-ray irradiator showing the horizontal dose distribution relative to the mean dose at the middle of the canister. The blue edge aroung the map is an artefact of the Excel surface plot routine.

and compacted in layers not greater than 5 cm. Over-compacting and stacking adults beyond this limit will reduce the quality greatly (Bouyer, 2020). Therefore, if using the full volume of the 2L canister (i.e. 250,000 *Aedes* adults), and a processing time (exposure time for ~60 Gy plus sample loading) of 12 min, 5 loads per hour can be irradiated. Therefore, around 75 million adult mosquitoes can be treated in a 5-day week, with two 6-h shifts. Due to the lower bulk density of adult mosquitoes (0.1 g cm^{-3}) the beam attenuation would be less and it is estimated that the DUR will be about 1.05.

X-rays, like gamma rays, have the benefit of high penetration, but unlike gamma rays, do not need a radioactive source for its generation. Although these types of radiation differ in their energy spectra, angular distributions, and dose rates, their effects on irradiated insects are substantially equivalent to those of gamma rays (Mehta, 2009). Bakri et al. (2021), in their review, stipulate that the types of radiation that are applicable in programs that release sterile insects include gamma rays, high energy electrons and X-rays, following several studies on insect dose response where no significant differences were found between X-rays and gamma rays. Some of these trials were carried out at the FAO/IAEA Insect Pest Control Laboratory in Seibersdorf, Austria, and irradiating male pupae of the same age of the South American fruit fly *Anastrepha fraterculus,* the melon fly *Bactrocera cucurbitae*, and the Mediterranean fruit fly *Ceratitis capitata* with the same nominal doses with gamma or X-rays had no significant effect on adult emergence rate and mating competitiveness of irradiated males competing for fertile females in walk-in field cages (IAEA, 2012; Mastrangelo et al., 2010). Sterility levels found in *Aedes* mosquitoes following irradiation in an X-ray irradiator were also found to be comparable to those following irradiation in a gamma-ray irradiator (Yamada et al., 2014).

5. Conclusion

In conclusion, the small dose variation within the irradiation chamber of the Raycell Mk2 X-ray blood irradiator, and the potential processing efficiency to sterilize reasonable numbers of mosquitoes and fruit flies indicate that this X-ray generator has promising application potential for SIT programmes against these, and potentially other insect species, and can serve as a cheaper and practical alternative to self-shielded gamma irradiators for insect sterilization in pilot and medium scale SIT programs.

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Declaration of competing interest

The authors declare that they have no conflicts of interests.

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Sterilizing insects with X rays or gamma rays - which irradiator to select?

Hanano Yamada[1*‡], Dongjing Zhang[2,3,4,5‡], Andrew G. Parker[1†] and Marc J. B. Vreysen[1]

[1]Insect Pest Control Laboratory, Joint Food and Agriculture Organization (FAO)/International Atomic Energy Agency (IAEA) Centre of Nuclear Techniques in Food and Agriculture, Vienna, Austria, [2]Chinese Atomic Energy Agency Center of Excellence on Nuclear Technology Applications for Insect Control, Key Laboratory of Tropical Disease Control of the Ministry of Education, Sun Yat-sen University, Guangzhou, China, [3]SYSU Nuclear and Insect Biotechnology Co., Ltd., Dongguan, China, [4]International Atomic Energy Agency Collaborating Centre, Sun Yat-sen University, Guangzhou, China, [5]Guangdong Provincial Engineering Technology Research Center for Diseases-Vectors Control, Sun Yat-sen University, Guangzhou, China

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EDITED BY
Riccardo Moretti,
Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), Italy

REVIEWED BY
S. Noushin Emami,
Stockholm University, Sweden

*CORRESPONDENCE
Hanano Yamada
h.yamada@iaea.org

†PRESENT ADDRESS
Andrew G. Parker,
Roppersbergweg 15, 2381 Laab im Walde, Austria

‡These authors have contributed equally to this work and share first authorship

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Introduction: what does ionizing radiation have to do with insect pest control?

There are thousands of insect pest species on this planet that damage crops and contribute to severe food insecurity and hunger in this world ( ). Other insect pests directly affect the health of livestock and humans, by transmitting parasites, bacteria, and viruses, and indirectly through the indiscriminate use of insecticides to manage these pests. These often very harmful chemicals leave residues in air, food, and water, causing significant health problems in humans, killing non-target and beneficial insects, and accelerate the development of insecticide resistance in the target insects and lead to outbreaks of secondary insects pests (,). It is estimated that 3.5 million tons of pesticides were used worldwide in 2020, at a cost of more than 60 billion Euros, and this highlights the need for insect pest control strategies that are more friendly to the environment and hence, more sustainable ( ).

In response to the global threat of insect pests and the harmful effects of insecticide use, the sterile insect technique (SIT) was conceptualized as early as the 1930's. It is a pest control tactic that is attracting more attention in all regions of the world, be it for implementation at small or large scale. The SIT is an autocidal control tactic that requires the mass-rearing of the target pest, their reproductive sterilization using ionizing radiation and sequential release into the target area for the reduction of the population with each generation ( ). Of crucial importance is the reproductive sterilization of the male insects as these must retain the ability to seek out and mate with wild females after being released, thereby inhibiting the development of viable offspring. Both high-energy particle and photon beams can be used to sterilize insects. Although particle beams (electrons, protons

and neutrons) have been tested, photons (cobalt-60 and less often cesium-137) are more commonly used for insect sterilization. However, X-ray irradiators have become more popular in the last decade, especially for use in smaller, or start-up SIT projects. The reasons are obvious, i.e., irradiation capacity can be established at a lower capital cost, simplified procurement procedures and the absence of safety regulations in the importing country. On the other hand, there is little information available on the long-term reliability (in terms of durability) of X-ray irradiators, an important prerequisite for SIT programs. In addition, concern has been expressed on the relative biological effectiveness (RBE) of X-rays as compared with γ rays to obtain the desired sterility in the irradiated insects, as well as the insect volumes that can be processed, especially for medium sized and larger programs.

A literature search revealed that very few scientific publications are available that describe X irradiation of insects and compare it with γ irradiation. Most of these are very old and precise handling and irradiation protocols are poorly described, if at all. Most of these reports tend in general to prefer X irradiation above γ irradiation in terms of RBE, as in most cases, a lower dose was needed with X-rays as compared with γ rays to achieve the same target sterility. But does this also imply that more somatic damage is induced in the insect overall? During recent studies, the importance of a parameter that was overlooked by nearly all older studies has become obvious, i.e., the significance of dose rate.

In this paper, we summarize the available information on X and γ irradiation for insects, with a focus on mosquito vectors, and discuss the advantages and limitations of both types of irradiation and revisit the importance of dose rate.

What are the differences between X-ray irradiators and gamma-irradiators?

Gamma- and X-rays are both high energy photons with ionization properties, but with different origins. Gamma-rays are emitted as a result of nuclear processes within radioactive isotopes and are all similar in energy (or a few energies), whereas X-rays are principally created by the deceleration of high energy electrons when they strike a target (Bremsstrahlung), which creates a full spectrum of photons from a maximum at the energy of the incident electrons down to zero energy, with few of the high energy photons and increasing numbers with decreasing energy. Both conventional orthovoltage X-ray tubes (150 – 320 kV) and electron beam accelerators (3-7.5 MV) with a suitable target produce X-rays. Several self-shielded X irradiators are manufactured these days, that have sufficient power and processing volume to be suitable for insect irradiation (–).

In the past, isotopic irradiators were predominantly used for insect irradiation because of their high initial dose rates, unmatched high reliability and, after installation, requiring only a modest electricity supply to operate (some like the Gamma Cell 220, can even be operated manually, in case of a power cut). Whereas panoramic irradiators have a good dose uniformity, small self-shielded gamma irradiators often have high dose variation within the sample chamber. The higher energy photons of gamma irradiators have greater penetration ability than those of low energy X-irradiators, allowing larger loads to be processed. The most significant drawbacks of gamma irradiators, however, are the security and regulatory requirements of importing and housing high activity radioactive sources. Finally, the high and increasing cost of cobalt-60 make them prohibitively expensive for some insect pest control programs and they require reloading every 5-20 years.

Self-contained X irradiators have the big advantage of no security issues and minimal regulatory requirements. Moreover, they have become less expensive than isotopic irradiators. However, the dose rate is much lower (typically between 3 and 15 Gy/min, and the process volume often smaller than many isotopic irradiators (panoramic gamma irradiators have a processing capacity of several billion insects per week, whereas self-contained X-ray irradiators are currently limited to millions of insects per week). They also require a reliable power supply (often 400 V) with attendant continuing electricity costs and they are complex systems, with the inherent reliability issues that go with complexity. A summary of characteristics of various irradiator types can be found in Table 1.

What is the significance of dose-rate in inducing sterility?

The discrepancies in dose-response that have been observed in different SIT programs with the same insect species and sometimes even with the same irradiators, compelled the initiation of a recent study to better understand the effects of dose rate in mosquito models. The study revealed an interaction between dose rate and dose ( ), but remarkably, the relationship proved to be non-linear, making the explanation of the mechanisms and causation of the biological effects very complex. Although the authors attempted a hypothesis to explain the interaction, the study concluded that dose rate is an important but neglected variable in insect irradiation and needs to be taken into account in SIT programs and reported in relevant publications. The data of the Yamada et al. study ( ) showed that at higher radiation doses (in the mosquito models used, the threshold was between 30 and 40 Gy), increasing dose rate resulted in a decrease in induced sterility, i.e., a diminished RBE and thus a shift of the dose-response curve to the right. Contrarily, at lower radiation doses (< 30 Gy), increasing dose rates resulted in increased RBE. The study was carried out with *Aedes aegypti* and *Anopheles arabiensis* (Dongola strain), and all insects were irradiated under very stable and consistent conditions in several Gamma Cell 220 irradiators, that had dose rates ranging from 0.4 to 79 Gy/min. What has yet to be studied is the extent of off-target effects (somatic damage) following radiation exposure at varying dose rates. Typical dose rates and energies related to different radiation sources are summarized in Table 1.

A detailed review of the historic reports that dealt with irradiation studies of *An. arabiensis* and taken into account only those that adequately reported dose rate and dosimetry, two clear scenarios support our findings that dose rate is a driving factor in dose-responses in insect sterilization: Figure 1A shows dose-

TABLE 1 Summary of typical characteristics of various irradiator types, their advantages and disadvantages.

	Gamma ray (panoramic)	Gamma ray (self-shielded)	Electron Beam (accelerator)	X-ray (high energy)[1]	X-ray (low energy)[2]
Characteristic					
Photon/ electron energy range (MeV)	^{60}Co 1.17, 1.33	^{60}Co 1.17, 1.33 ^{137}Cs 0.66	1 – 20[3]	1 – 7.5[4,5]	0.15 – 0.225[5]
Emission pattern	Isotropic	Isotropic	Narrow beam (~1 cm dia.) scanned across conveyor belt	Narrow beam (~1 cm dia.) scanned across conveyor belt	Isotropic
Penetration (cm in water to half dose rate)	High (20/23)	High (20/23)	Low (2-7)[6]	Very high (15 – 40)	Low (5 – 10)
Typical dose rate (Gy.min^{-1})	Low-medium (5 – 20)	High (20 – 300)	Very high (100 – 10000)	Medium (5 – 500)	Low – medium (3 – 15)
Process method	Continuous/batch[7]	Batch	Continuous[8]	Continuous[8]	Batch
Process time	Long	Short	Very short	Short – medium	Medium – long
Throughput (million fruit fly pupae/hr at 100 Gy)	6 – 12	0.2 – 0.8	0.05 – 19	0.08 – 54	0.025 – 0.37
Advantages and disadvantages					
Advantages	High throughput; Low running costs; Good dose uniformity; Very reliable; Capital costs lower than accelerator; Can be incorporated into a conveyor/automated system	Low running costs; Fairly good dose uniformity; Very reliable; Lower capital costs; No external shielding required	Can be integrated into automated system (conveyor belt feed); Licensing easier than cobalt	Can be integrated into automated system (conveyor belt feed); Licensing easier than cobalt; Good penetration allows load to be irradiated in transport containers	Cost effective options available; No shielding required; Redundancy by procuring two; Simple licensing and regulation
Disadvantages	Source replacement costs high; Radiological safety issues; Strict regulations on procurement and transport; Shielding required; Security costs (human resources and equipment)	Source replacement costs high; Radiological safety issues; Strict regulations on procurement and transport; Security costs (human resources); Difficult to integrate into automated system; Increased labour and handling	High capital and running costs; Requires shielding; No redundancy; Requires qualified service technicians; Very low penetration requires insects to be in shallow trays; Load must be flipped or two opposing accelerators to get adequate dose uniformity; Very high dose rate requires high conveyor speed	High capital and running costs; Requires shielding; No redundancy; Requires qualified service technicians; Load may need to be flipped or two opposing accelerators to get adequate dose uniformity; High dose rate requires high conveyor speed	Difficult to integrate into automated system; Increased labour and handling; Limited reliability record; Time and cost to replace X-ray tube; Requires qualified service technicians

[1]High energy X-rays are generated from an electron beam striking a suitable target material.
[2]Low energy X-rays are mostly generated by conventional orthovoltage tubes.
[3]Electron beams often have a single fixed energy for a given accelerator.
[4]X-rays are normally limited to a maximum of 7.5 MeV to prevent neutron activation.
[5]These energies are the peak photon energy. The effective energy of the X-rays, the mean energy of all photons produced, is approximately one third of the peak energy.
[6]The penetration of electron beams does not follow the near exponential decay of a photon beam. The beam intensity falls very rapidly to zero beyond the half dose rate point.
[7]Panoramic gamma irradiators can be arranged for batch processing for smaller units, with a number of fixed turntables, or continuous processing for larger units, with a conveyor system.
[8]Electron beam and high energy X-ray systems require the load to be moved under the scanned beam on a conveyor belt.

response curves of *An. arabiensis* (Dongola) irradiated in the same Gamma Cell 220 over a period of 12 years with dose rates of 16, 93, 84 and 74 Gy/min (low activity at the onset, high activity after reloading, and normal decay thereafter). A dose-response curve following X irradiation of the same strain is plotted for comparison and indicates that lower doses were required to reach the same target sterility. However, the X-ray irradiator dose rate is also the lowest in this comparison (Figure 1A, curve [a]). In Figure 1B, dose-response data for *Ae. aegypti* males irradiated in various X- and gamma irradiators show a decrease in biological effects with increasing dose rates, irrespective of radiation source, clearly indicating that dose rate is a more important factor than source

FIGURE 1
(A) Published dose-response curves (induced sterility against log (Dose) for *Anopheles arabiensis* (Dongola) irradiated with X-rays (Raycell, MK2), compared to γ irradiation with a GC220 with increasing dose rates. [a] (10); [b] (11); [c] (Ntoyi, unpublished data[1]); [d] (9); [e] (9); **(B)** Dose-response curves of *Aedes aegypti* irradiated in various irradiators (X and γ) with increasing dose rates. [f] (10); [g] (Juarez, unpublished data[2]); [h] (12); [i] (13); [j] (14); [k] (15).

type. Very few reports are published that compare the RBE of X- and γ-radiation directly in the same species, and in which other factors that affect dose response are controlled and accounted for. Even fewer adequately describe irradiator characteristics, especially the dose rate, and report dosimetry. The results of these studies are therefore ambiguous, confusing and questionable.

Discussion: X-ray versus gamma-ray: which is preferred?

From a biological point of view, source type does not seem to influence irradiation outcome as much as dose rate and other potential biological and physical factors that play a role in dose-response (9, 16, 17). The debate should, therefore, not focus on which radiation source is better or worse, but rather be centered on the prerequisite to create awareness that dose rate is a very critical factor in dose-responses for insect sterilization, especially in operational action programs. These SIT programs should incorporate in their quality control protocols regular checks on the dose responses of the insects that are destined for release. Overdosing will reduce the quality of the released insects and underdosing will induce a lower sterility in the target female population. The outcome for both scenarios will be reduced efficiency of the program and success doubtful. This is especially crucial for programs that use gamma irradiators in view of the natural decay of the source and resultant change in dose rate.

The selection of X- or gamma ray will most like be dictated by the type or size of the program, i.e., economics rather than biology. For smaller, start-up programs, and where infrastructure and electricity supplies are suitable and reliable, the cheaper X-ray machines are probably the preferred choice. Industrial, panoramic or larger self-shielded gamma irradiators will remain indispensable for large operational SIT programs that require high output, utter reliability and sustainability.

Other options might become available in the future such as electron beams from linear accelerators. These potential competitors that are on the horizon are currently not commonly used in SIT programs. They are large, complex and expensive machines and the only option currently viable is to purchase the service from a commercial supplier. Smaller, compact accelerators with dose rates more suitable for insect irradiation are under development and the first prototypes might become available on the market in a couple of years. They are likely to still be relatively expensive but may be an alternative option for larger programs.

Author contributions

DZ proposed the discussion and topic. HY and DZ developed the discussion and drafted the initial manuscript. AP and MV contributed significantly to the discussion and the development of the paper and final draft. All authors contributed to the article and approved the submitted version.

1 Nonhlanhla lindiwe Ntoyi <lindiwe.ntoyi@gmail.com>. The data presented was collected at the IPCL, IAEA.

2 Jose Juarez <jjuarezvaldez@gmail.com>. The data presented was collected at the IPCL, IAEA

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Conflict of interest

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Publisher's note

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Nuclear Techniques in Food and Agriculture

Guidelines for Irradiation of Mosquitoes in Sterile Insect Technique Programmes

Version 1.0

Food and Agriculture Organization of the United Nations
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Guidelines for Irradiation of Mosquitoes in Sterile Insect Technique Programmes

Edited by:

Hanano Yamada, Andrew Parker, Hamidou Maiga and Jérémy Bouyer.
Insect Pest Control Section, Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture

Cover photo credit: Hanano Yamada

Food and Agriculture Organization of the United Nations
International Atomic Energy Agency
Vienna, 2020

FOREWORD AND AKNOWLEDGMENTS

This publication is intended as guidance for the irradiation of mosquitoes at pupal and adult 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 and adults, 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 and adults 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.

Table on Contents

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 environment-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".

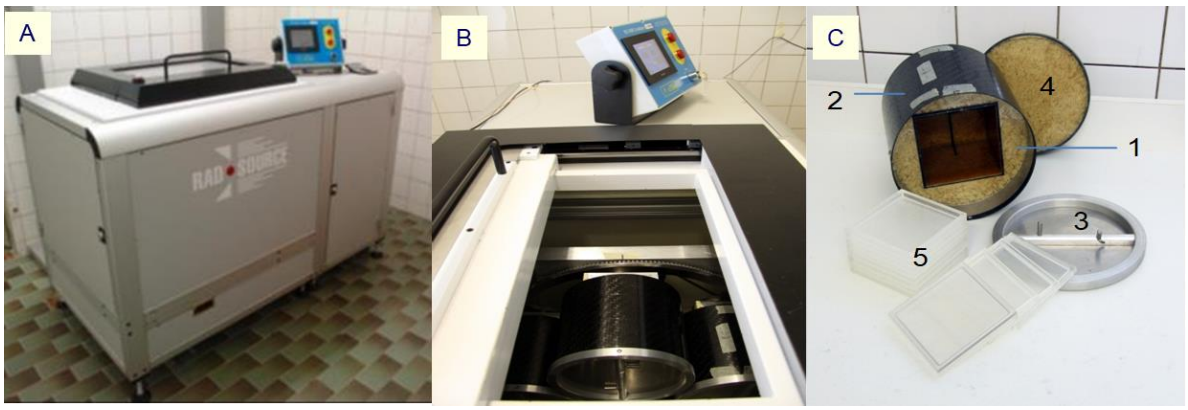

Figure 2. The RadSource 2400. A) the irradiator (cooling system not shown), B) the chamber with 5 rotating canisters with processing capacity of 200,000 Aedes *pupae, and C) a holding canister consisting of 1. Plexiglass cylinder (filled with rice), 2. Carbon fiber canister, 3. Aluminium lid, 4. Plexiglass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

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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)".

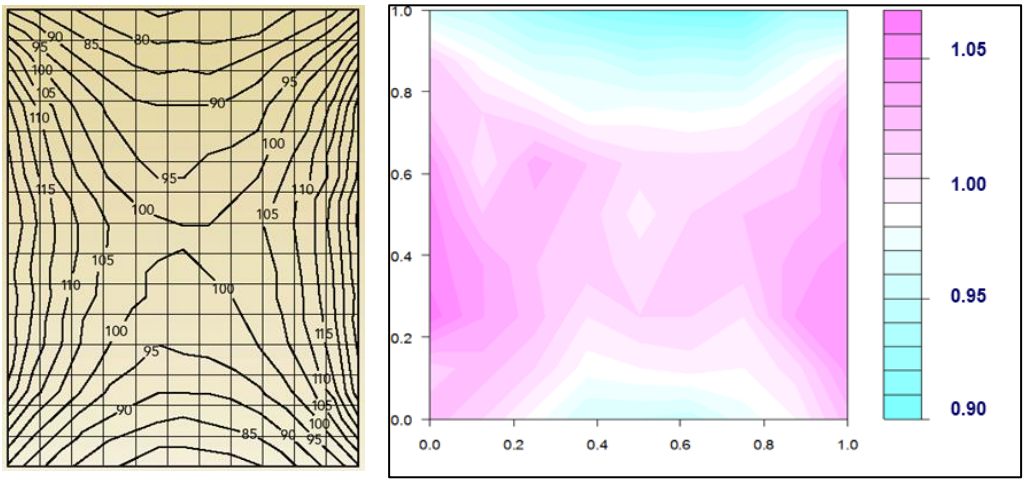

Figure 3. Dose distribution maps. A) a vertical section dose map of a GC220, with doses varying from 75-135% of the center dose (DUR= 1.8), and B) Dose distribution map of a rotating canister in a RadSource 2400 with a -10% and +7% from the center point.

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".

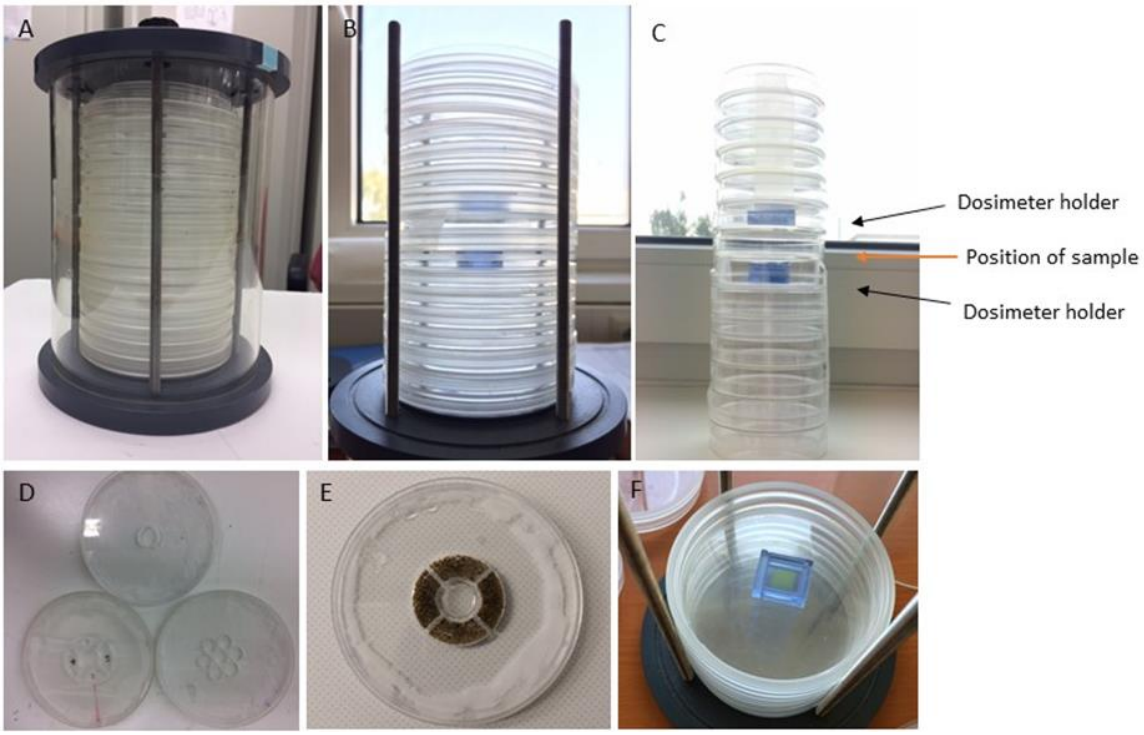

Figure 4. Holding containers for the standard irradiation of mosquito pupae at laboratory scale. A) custom made canister made of stacked petri dish (without lids) held together by plastic rings, surrounded by a Plexiglass tube to provide adequate build-up material. B) Central position of the sample and 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.

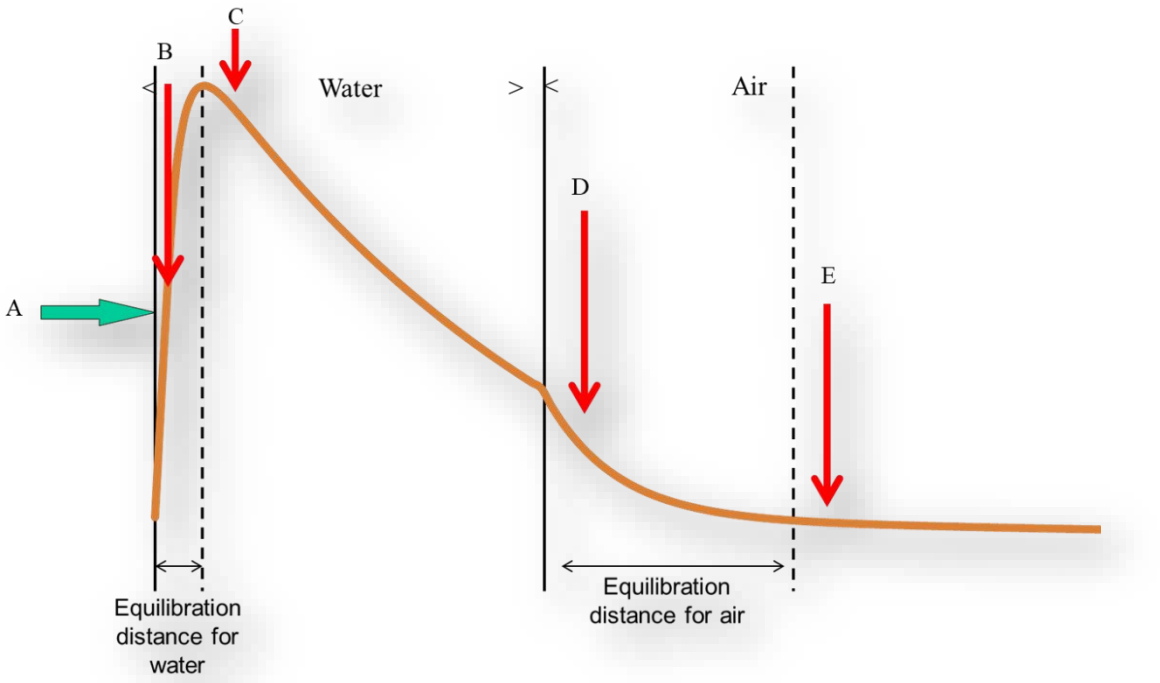

Figure 5. The importance of build-up material in irradiation. Canister material (and its density) and wall thickness are important factors.

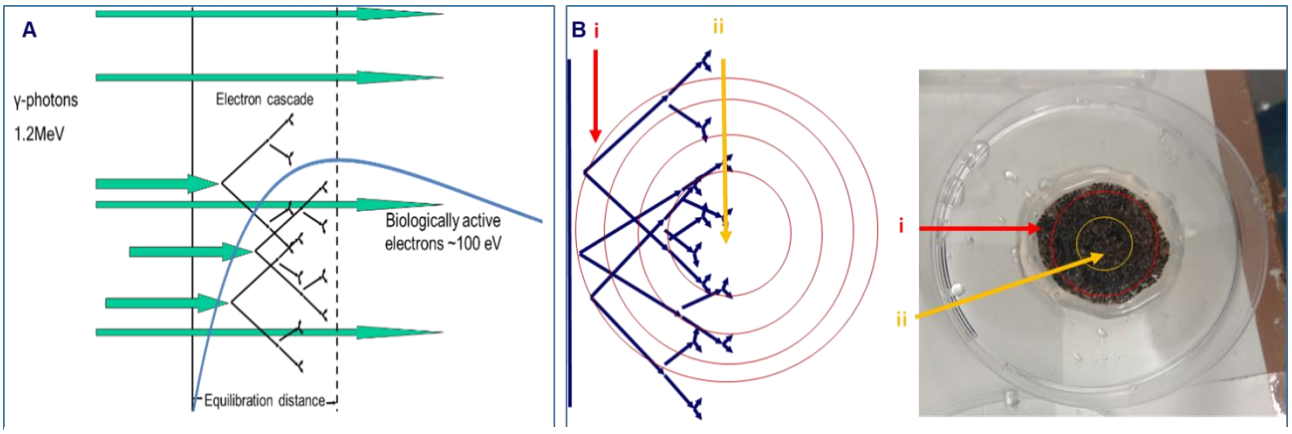

Figure 6. The equilibrium distance and electron cascade in exposed samples. A) graphical presentation of the electron equilibrium in the presence of build-up material. B) Effective dose received by a monolayer of pupae positioned in the center of a petri dish in the absence of surrounding build-up material. 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].

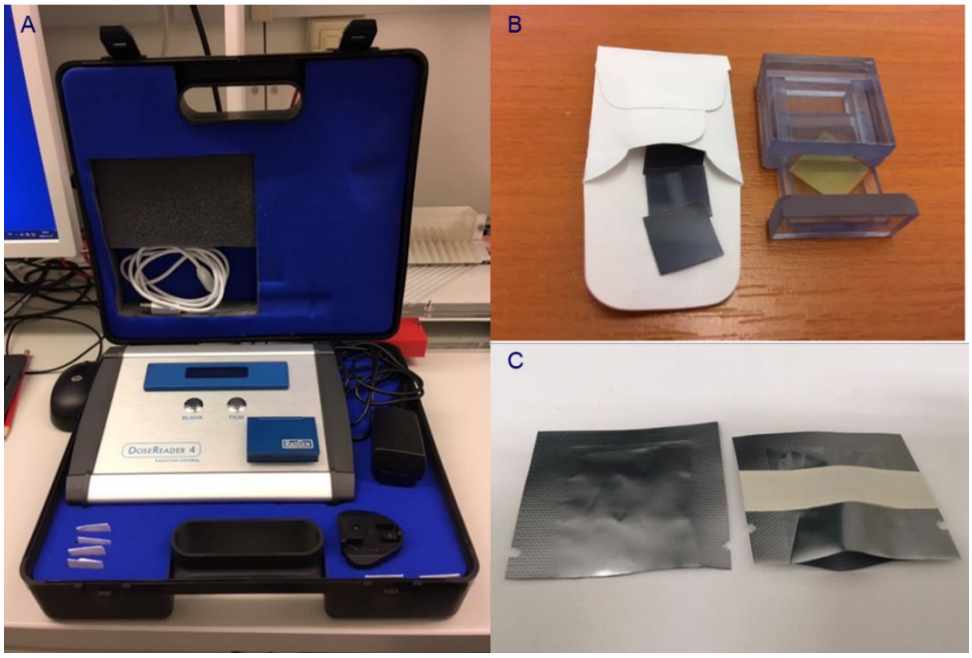

Figure 7. Gafchromic dosimetry system. A) the optical density film reader, B) paper envelope containing 3 exposed films (left), and a plastic dosimeter holder containing 1 unexposed film, and C) aluminium pouches that can be heat sealed, with an adhesive strip for sticking the envelope onto a container.

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.

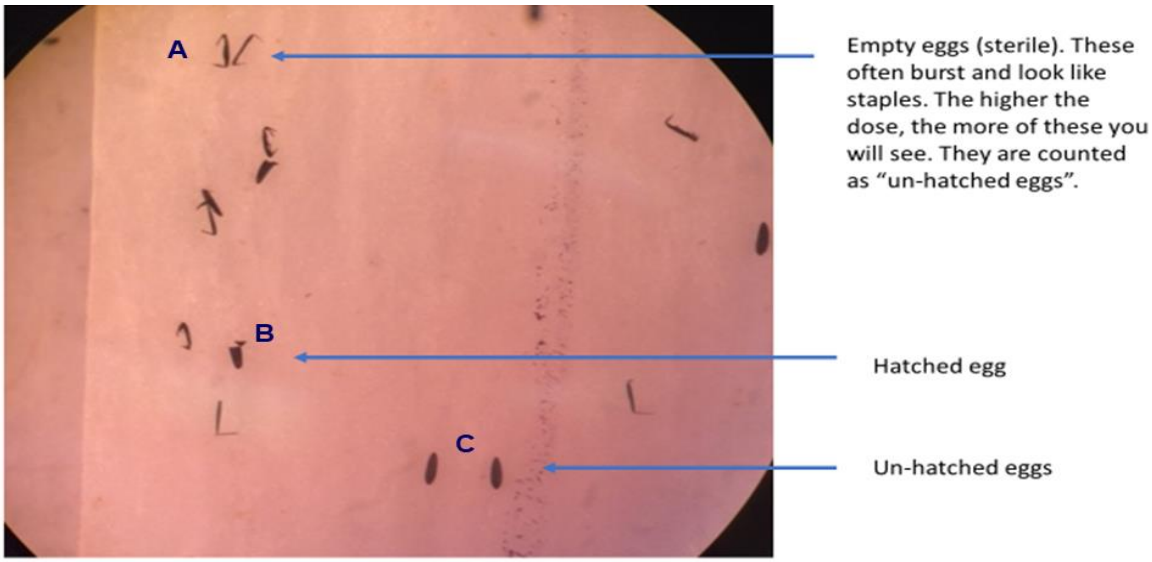

Figure 8. Counting hatched and un-hatched eggs under the stereomicroscope. A) unfertilized, empty eggs that have burst, having an appearance like a staple, B) hatched eggs with detached egg cap, and C) intakt, unhatched eggs, which may or may not contain an embryo.

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.

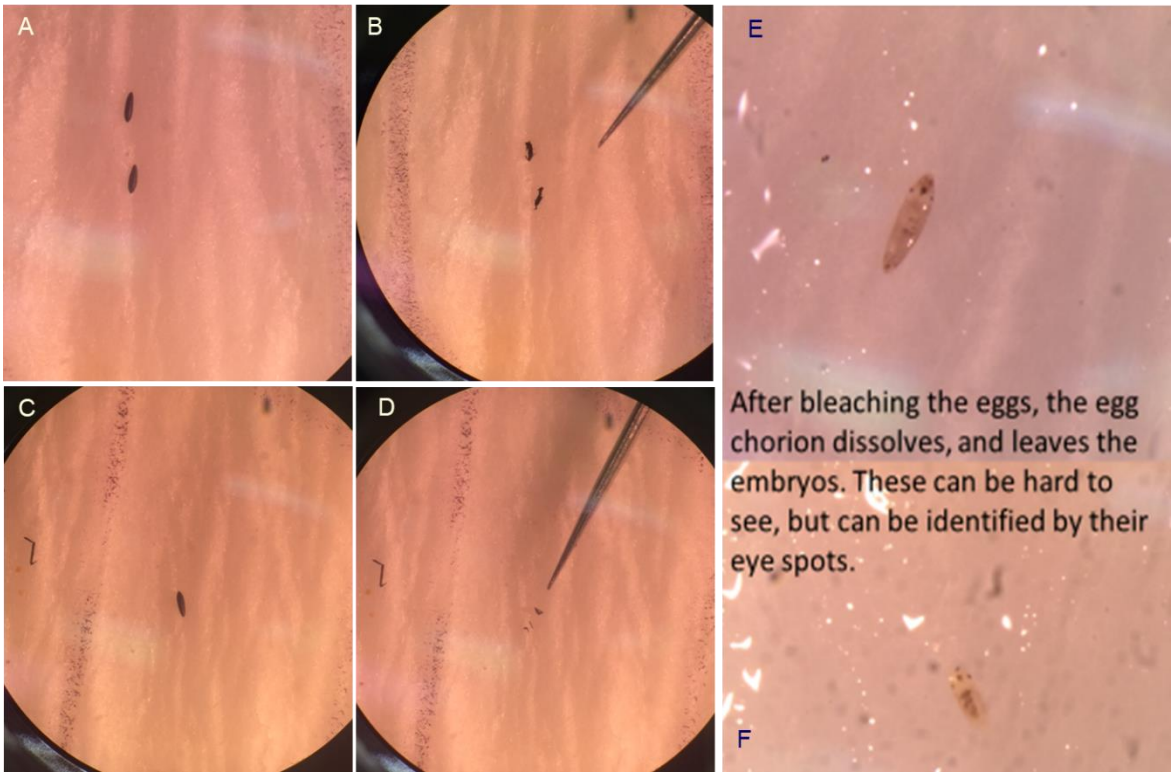

Figure 9. Determining fertility status of unhatched eggs. A) eggs appear to be unhatched and intact. B) the same eggs after being burst with a dissection needle. C) Intact egg. D) egg after burst with a dissection needle, giving an appearance of a staple. A-D) these intact eggs are all empty, and therefore 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 been shown in mosquito pupae, in which chilling slightly reduces radiosensitivity in Aedes spp. (Yamada et al 2022). 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

The dose-rate affects irradiation outcome in both mosquito pupae and adult stages. The interaction of dose and dose-rate is complex, and it is important to implement biological dosimetry periodically as a QC measure to ensure that the sterility achieved reaches the desired levels, when gamma sources decay over time, or are reloaded, and thereby changing the dose-rate of the irradiator.

5. Irradiating mosquitoes at adult stage

Standardizing irradiation protocols for pupae is difficult, especially in practical terms in large-scale SIT programmes for the following reasons: Pupal age is an important factor that significantly impacts dose-response [6,26]. Although guidelines exist for the optimization of larval rearing for synchronized pupae development [27], it is in reality unrealistic to narrow the pupation window to 16 hrs or shorter, to ensure that all pupae are aged 30 hrs or older during the irradiation process. Also, timing the pupation so that the collection, sexing and irradiation can occur during daytime working hours is another challenge. Irradiating mixed age batches is not recommended, as irradiating younger pupae can negatively affect adult quality [6], and over-dosing (as younger pupae require less dose) would further exacerbate this. Conversely, irradiating younger pupae at an optimal dose (to achieve >99% sterility) is possible, however the risk remains that older pupae would be under-dosed, leading to potentially releasing significantly sub-sterile males, on top of males with diminished quality, thereby compromising success of the otherwise effective SIT. Additionally, and equally problematic is that it is difficult, if not impossible to control the atmospheric conditions surrounding pupae during irradiation in bulk. For mass irradiation at the pupal stage, the pupae would need to be placed in sufficient water within the irradiation canister to provide buoyancy to avoid the pupae at the bottom being crushed. However, this creates a hypoxic environment as pupae submerged in water continue to respirate through their cuticle and quickly deplete the surrounding water

of dissolved oxygen [28]. As hypoxia reduces irradiation effects, the irradiation of pupae in water results in differential levels of sterility within the sample [28], therefore this method for irradiation cannot be reliable unless, again, the full cohort is significantly overdosed. Apart from quality costs of over-dosing, pupae exposed to hypoxia suffer additional stress and loss in quality. Large numbers of pupae can also be irradiated without water in monolayers, however, pupae are still closely packed and pockets of hypoxia still occur within the sample (Louis Clement Gouagna personal communication) resulting in a proportion of pupae maintaining unacceptable levels of fertility. Drying pupae and spreading them in a manner that would avoid these issues is simply not practical at large scale and is expected to incur detrimental levels of stress to the pupae.

It is for these reasons, that the irradiation at adult stage has been considered as a more practical and reliable option for the bulk sterilization of mosquitoes. Most notably, water, and thus hypoxia would no longer be a variable factor

Selecting adult age

The significance of adult age in terms of irradiation induced sterility has yet to be assessed. However, for the preservation of production efficiency of the SIT, it is not feasible to store adults for prolonged periods of time, and sterile males need to be released at their peak flight and mating activity to enhance their beneficial effects in the field. As recovery post irradiation/pre-release is an important factor for adult quality (in terms of flightability), and sterile males should ideally be released aged 3-5 days (Maiga et al., submitted), irradiation should take place in adult males aged 1-2 days old. (Data on age effects on dose-response and quality are pending).

Preparing adults for irradiation

Adult mosquitoes can be irradiated (at small scale) in a non-immobilized state. However, it is important to note that adults moving around in a confined space will damage each other and will also change position within the container and may receive varying levels of radiation dose. Therefore, if the exposure in an awake state is necessary, received dose and uniformity between individual adults within and between samples will increase as the container size in which they are held is decreased. Also ensure that the placement of the sample within the irradiation chamber stays consistent.

An easy way to irradiate awake adults is to transfer them into a plastic "Drosophila" tubes (9cm height x 2.7cm diameter) with an aspirator and closed with a sponge. The sponge can then be pushed down before irradiation to minimize the space in which the sample is confined to (figure 1a).

Adjusting samples to comparable positions within the irradiation chamber is also necessary when, for ex. comparing two treatments (ex. Figure 1, a & b), to ensure that all samples are receiving the same dose. Remember to ensure that there is sufficient build-up material around the samples during radiation exposure (see "Build-up material" on p. 17 in this manual).

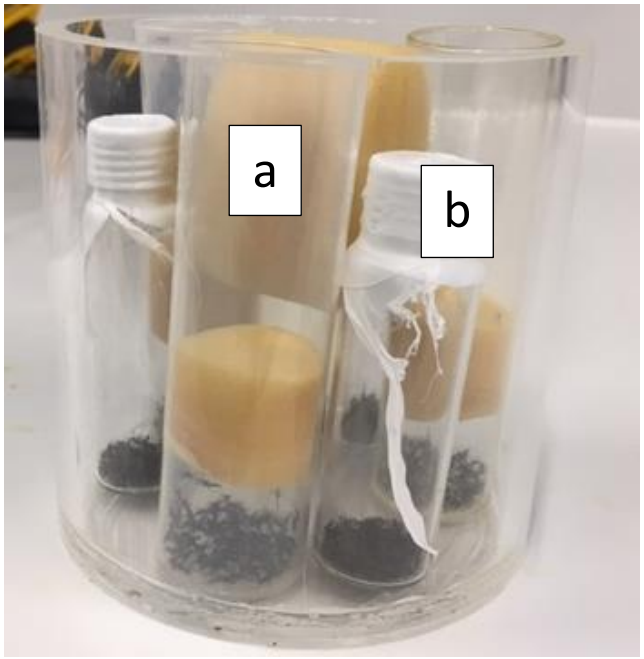

Figure 1. Samples of adult mosquitoes treated in either air (a) or immobilized in nitrogen (b) arranged around the center of the holding canister for uniform dose.

Immobilization

The irradiation of larger numbers of mosquito adults requires anesthetics for facilitating handling, and to minimize insect damage by their movement in confined spaces and especially when compacted for mass-irradiation. There are numerous methods to immobilize mosquitoes and other insects:

Chilling. Chilling is practical as it does not require the application of chemicals/gases. However, chilling can have negative effects on adult quality if the applied temperatures are too low, or

treatment durations too long [29,30]. No adverse effects on survival were reported for *Anopheles arabiensis* after chilling at 2,4,6 and 10°C, for up to 8 h [29]. Only chilling at 2°C for 24 h resulted in a decrease in longevity in this species. The optimum chilling temperature and duration for *Ae. albopictus* was found to be 5-10°C for 3 h, resulting in no adverse effects on longevity and mating competitiveness [30]. Adults are more sensitive to treatment in cold temperatures than pupae, and longevity and flightability were negatively impacted in *Ae. aegypti* following chilling at 7C for 2 hrs prior to, and during irradiation. It seems, however, that adverse effects resulting from chilling on flight ability can be, to an extent, reversed after allowing a recovery phase of 1-2 days (Yamada 2022, Maiga, 2022).

Nitrogen. Nitrogen can also be used to immobilize mosquitoes for handling and irradiation processes. The knock-down times are very fast in mosquito adults and is thus convenient for fast preparation. The wake-up times, however, increase as treatment duration increases, and therefore it is necessary to plan treatment durations accordingly. Aedes spp have a higher tolerance for treatment duration in Nitrogen (anoxia in general) [28] than *Anopheles arabiensis*. However, deleterious effects are seen in all species after a duration threshold and needs to be determined for each species.

Other. Several other methods to anaesthetize insects include CO2, argon, chloroform, desflurane, ethyl ether, triethylamine etc. [31–33].

Many reports have shown that immobilizing agents can have an impact on irradiation dose-response (see later sections), and can induce negative side effects [34,35], the extent of which depends on several biological characteristics of the insect, as well as the duration and frequency of exposures to the various gases, similar to treatments in cold temperatures. It is therefore necessary to carefully assess these factors and all available options before formulating protocols for mosquito immobilization and handling.

Consistency in environment, materials and methods

Irradiation container size, shape and material are also important for consistent, reliable, and uniform adult sterilization (see previous section, p.13, p. 16 of this manual). If cold packs i.e. phase change material is used for keeping adults immobile for longer exposure times, it is crucial to add these to the irradiation canister without unintentionally attenuating the irradiation beam. If the gamma ray source or x-ray beam is provided from above/below, put the cold packs on the sides of the canister, or ensure that the exposure time is adjusted to maintain the desired

absorbed dose. Similarly, if the radiation source is provided from the back/front/around the sample chamber, place the cold packs above/below the samples.

Routine dosimetry

Dosimetry should be performed with each sample irradiation to verify the absorbed dose, as described earlier in this manual (p. 19). As the density of adult mosquitoes is closer to air than water (as found in pupae), the calibration of the dosimetric films shall be completed in air (see Guidelines for Dosimetry IAEA) [36]. Again, the dosimetric films should be placed as close to the sample as possible, with sufficient build-up material surrounding the sample and the films (see p. 17 of this manual).

Factors that affect dose response in adult mosquitoes

As seen in pupae irradiation, there are several factors that affect dose response in adults. These can be grouped in biological or physical factors. Biological factors include the insects' intrinsic characteristics such as age, sex, life stage, nutritional state, etc. Physical factors include external variables such as ambient temperature, atmosphere, the dose-rate of the irradiator.

Developmental stage

In both *Aedes spp (Ae. albopictus and Ae. aegypt*i), and *An. arabiensis*, adults were slightly more radiosensitive than late stage pupae [2,37] (Yamada et al submitted). In many experiments, the difference was not more than ~3% induced sterility, and often non-significant. However, one must consider that the younger the pupae, the more sensitive and the more prone to somatic damage [26].

Age

The effects of adult age, if any, will be determined and added as results are obtained.

Sex

As seen in mosquito pupae, and most other arthropods, females are more sensitive to irradiation than males. See p. 28 of this manual for more details.

Ambient temperature

Chilling is a convenient method for immobilizing adults mosquitoes for easier handling, such as for irradiation procedures. Cold temperatures seem to have a slight radioprotective effect in mosquito adults in terms of sterility induced. In *Ae. albopictus*, chilling reduced overall radiosensitivity and thus sterility by about 3% when chilled at 7C° for 2 hrs prior to, and during irradiation. However, downstream quality parameters were negatively affected for several days before a partial recovery was observed (Yamada et al 2022). This may be the consequence of the reduced metabolic rates in the mosquitoes, whereas the increased somatic damage leading to reduced adult quality in the parameters assessed may be explained by higher oxygen saturation in the low temperature, leading to an increase in oxygen-dependent effects of irradiation, as proposed by Langely and Maly [38]. In any case, it is known that both radiation damage as well as recovery are temperature-dependent and are both slow in cold temperatures [39].

Few reports exist on the direct effects of chilling on irradiation dose response. Usually only one cold temperature is tested. It is unclear whether various temperatures (cold and warm) have differing effects. In any case, when new irradiation protocols are developed, biological dosimetry should be performed to ensure that the samples are reaching the expected sterility levels.

Ambient atmosphere

As in mosquito pupae and other insects, low oxygen environments are highly radioprotective and dose response, thus induced sterility can be reduced significantly following irradiation in hypoxia and anoxia. (see p. 29 of this manual for more details).

Dose-rate

The dose-rate of irradiators can differ between devices or can change over time due to the source’s natural decay. Although small changes in dose-rate may not affect irradiation outcome, it is important to note the there indeed exists a dose dependent dose-rate effect in irradiation in mosquitoes (Yamada et al 2022 and references therein). It is therefore essential to confirm sterility levels periodically as the isotopic source decays over time, or when the irradiator is changed to, for example, an x-ray irradiator with a different dose-rate.

Recovery time

A recovery phase after irradiation can significantly improve select biological quality parameters. For example, a reduction in flight ability is often observed right after, and 1-day post-irradiation. However, flight ability is often completely restored if the sterile males are allowed a recovery phase of 2 days (Maiga et al 2022). The capacity to fly also directly affects mating propensity, and thus a recovery phase before field release is recommended for *Aedes spp*. This has yet to be confirmed for other species.

Establishing dose-response curves for adult stage mosquitoes

Determining fully sterilizing doses and establishing dose-response curves for adult mosquitoes is in principle the same as for pupae.

Protocol for the establishment of a dose response curve for adult mosquitoes (Appendix III)

Up-scaling samples size towards mass irradiation of adult mosquitoes

There are some factors to consider when up-scaling the number of mosquitoes for irradiation. First, the development of a new canister/container will become necessary. As the container size will increase, the DUR is likely to increase. According to the dose distribution map of the container, a cost-benefit analysis should be done to decide whether higher/lower dose areas within the canister should be omitted to reduce the number of over- or under dosed males. This may not be necessary if sterile males are abundant and competitive, and high processing capacity is prioritized.

Immobilization of the adult males is required to avoid damage of the mosquitoes when handling and packing large number of adults for irradiation, and prior/subsequent transportation. This can be achieved by exposing adults to low temperatures or to anaesthetics such as CO2 (Poinapen et al., 2017), chloroform or desflurane (Cevik et al., 2019), ethyl ether or triethylamine (Cabrini et al., 2016) or nitrogen (Hallinan & Rai, 1973). The appropriate choice depends on available facilities, and on downstream effects of the methods on male quality parameters.

6. 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 mosquitoes produced for the SIT" *(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	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. **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.

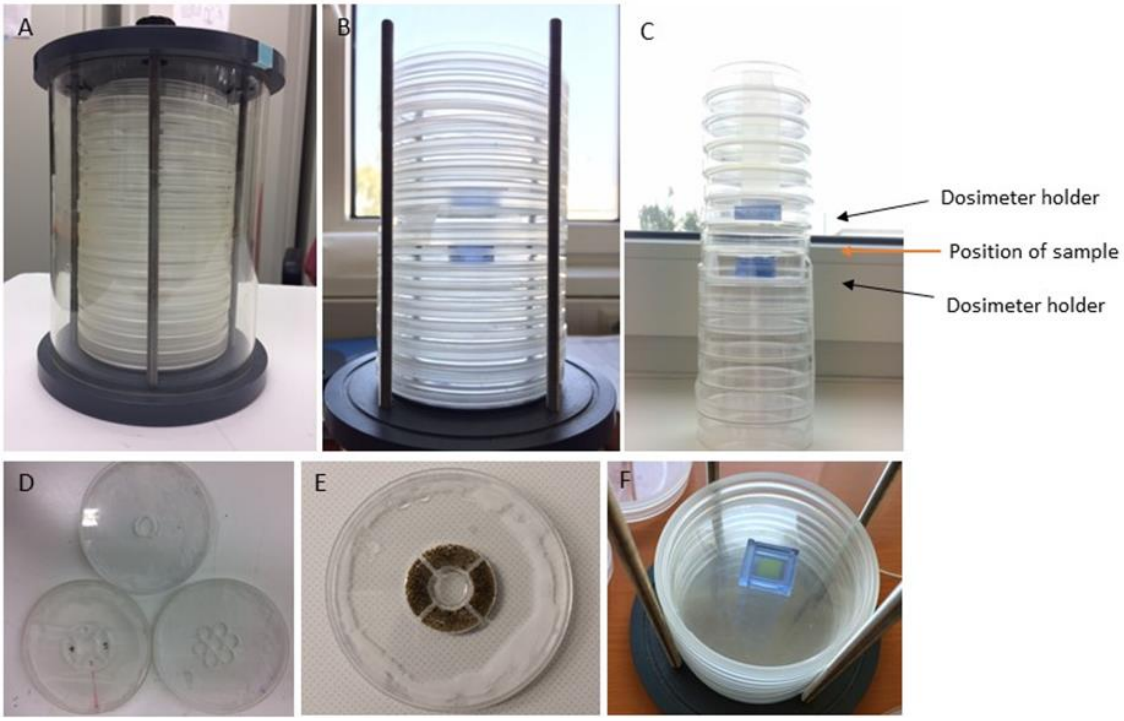

Figure 1. Examples of purpose-made petri dish holders for the standardized irradiation of mosquito pupae. Pupae are placed in the middle of the dish (Figures D & E), and dosimeters above and below the samples (figures B,D & F). Adequate build-up material is provided by a >4mm PMMA surrounding the sample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

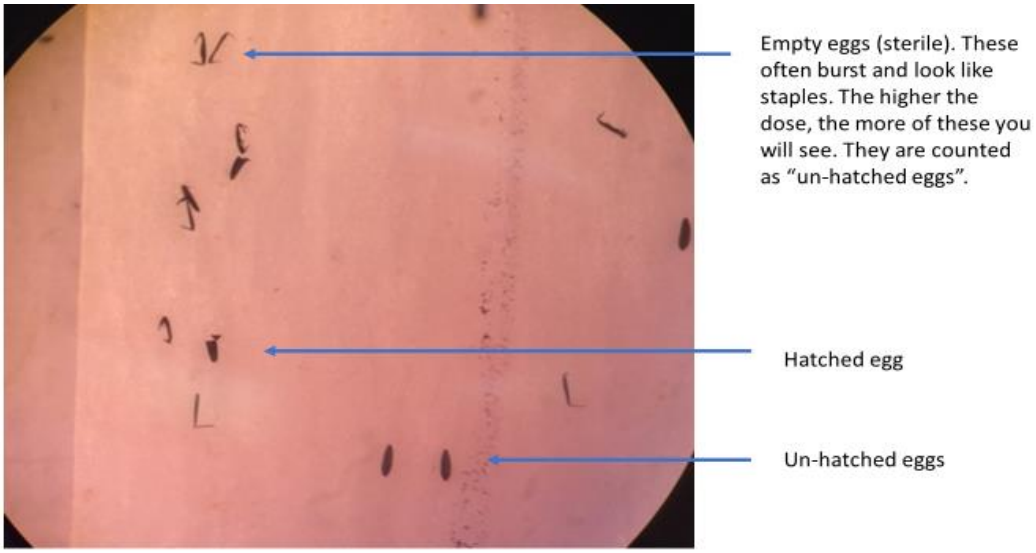

Figure 3. Hatched, un-hatched, and empty/burst eggs.

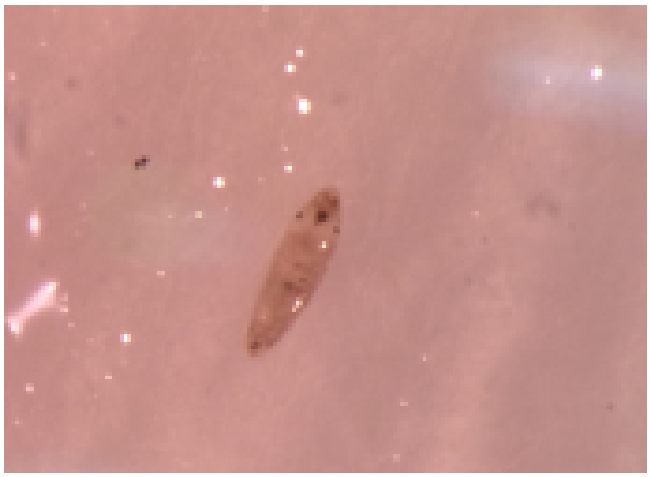

Figure 4. Determining the presence or absence of embryos. After bleaching the eggs. The egg chorion dissolves and leaves the embryos. These can be hard to see, but can be identified by their eye spots

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.
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.

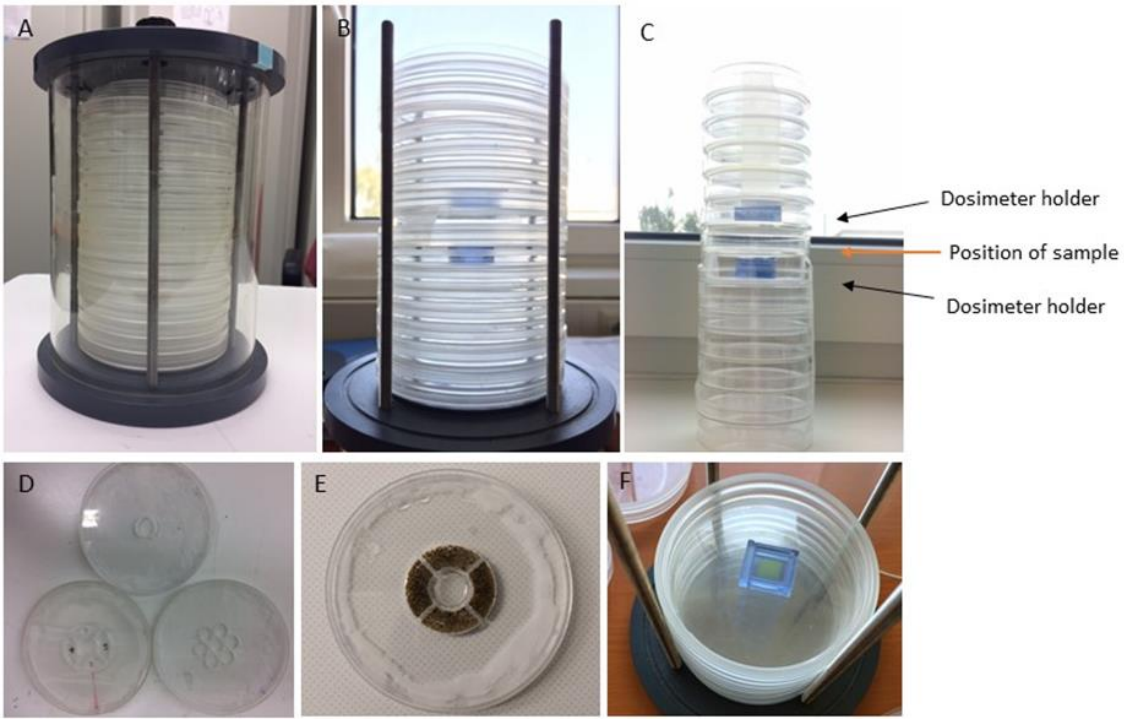

Figure 1. Examples of purpose-made petri dish holders for the standardized irradiation of mosquito pupae. Pupae are placed in the middle of the dish (Figures D & E), and dosimeters above and below the samples (figures B,D & F). Adequate build-up material is provided by a >4mm PMMA surrounding the sample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

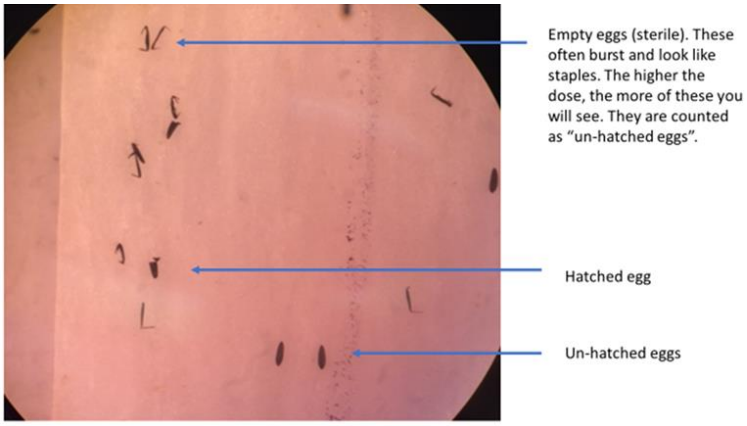

Figure 2. Hatched, un-hatched, and empty/burst eggs.

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

Appendix III: Protocol for the establishment of a dose response curve for adult mosquitoes

1. **Rearing:** Rear immature stages following the guidelines for the Routine Colony Maintenance of *Aedes* spp. Try to synchronize hatching and rearing to get good proportion of pupation and thus adult emergence on the first day of the adult stage.

Rear at low densities (~2 larvae/ml) to ensure good pupal size and male/female size difference if sexing is planned at pupal stage.

2. **Collect pupae**: Collect pupae on the peak pupation day to get as many adults emerging on the same day as possible. All adults for the experiment should be of similar age.

3. **Sexing**: sexing can be done at pupal stage or at adult stage after emergence. Sexing and quantifying pupae is usually faster and easier. It is also recommended as virginity can be ensured if females are isolated already at pupal stage. The desired number of males can then be aliquoted into separate cages or containers for emergence and subsequent irradiation.

4. **Keep female pupae**: 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 adding them to males.

Materials, doses and replications:

- Strain: for example, *Aedes aegypti* (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 adults: >1080**		**540 males**	**540 female**

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.

6. **Irradiation**: For each replication, place the groups of 30 male adults in a "drosophila tube" or other tube-like container. (It is just important that the technical (pseudo) repetitions are irradiated at the same time and can be arranged symmetrically within the irradiation chamber to all receive a similar dose). The adults should be contained to a restricted space within the container and thus irradiation chamber. Therefore, using sponges that can be pushed down the tubes just before irradiation are convenient (figure 1a). Ensure that the mosquito samples are surrounded by sufficient build-up material (see p.17 of this manual for more information about build-up material). The place the container with the samples in the middle of the irradiation chamber. Add your dosimeters near your sample. Remove the sample from the irradiator after exposure, and return the males to labelled cages with sugar feeders. Repeat with all reps, for all doses.

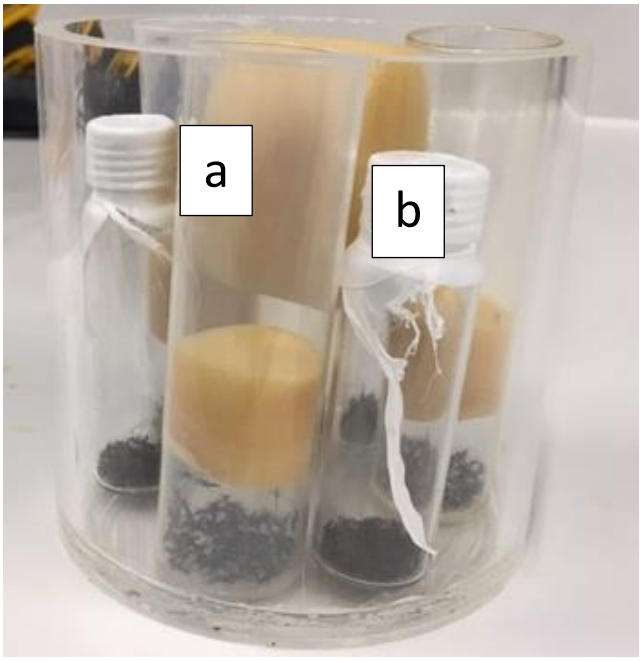

Figure 1. Samples of adult mosquitoes treated in either air (a) with a sponge holding adults within a restricted space, or immobilized in nitrogen (b) arranged around the center of the holding canister (with 4mm PMMA walls for sufficient build-up material) for uniform dose.

7. **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).
8. **Blood feeding:** Blood feed the cages on 2 consecutive days (day 4 and 5 post-emergence).
9. **Oviposition:** add oviposition papers in labelled cups and 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).
10. **Egg maturation, storage and hatch:** See Appendix I of this manual for Aedes spp egg storage and hatching, and Appendix II for Anopheles egg hatching.
11. **Record data**: 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.
12. **Bleach eggs**: To check the un-hatched eggs for the presence of embryos you can puncture the unhatched eggs with a dissection needle. If there are many unhatched eggs in *Aedes spp*., it is convenient to 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).

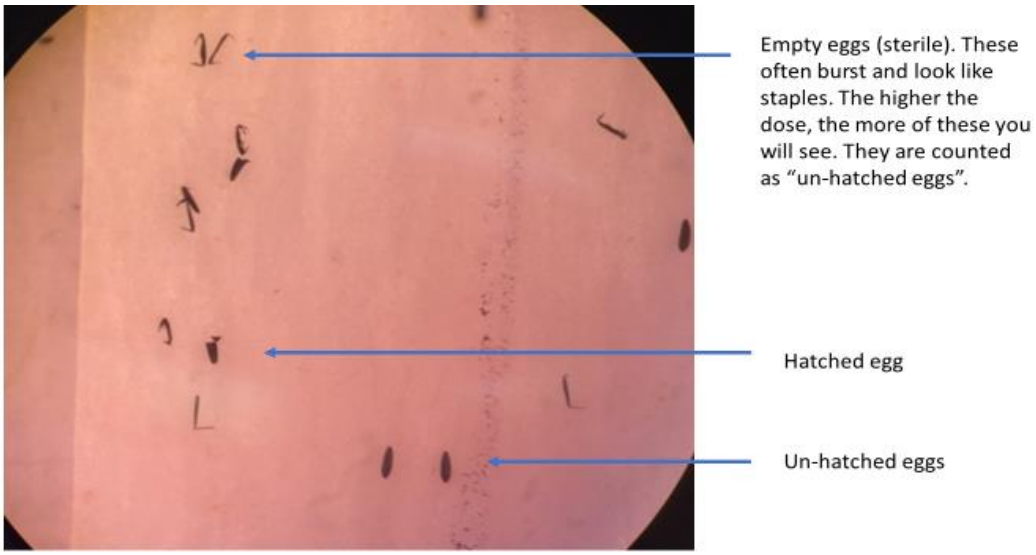

Figure 3. Hatched, un-hatched, and empty/burst eggs.

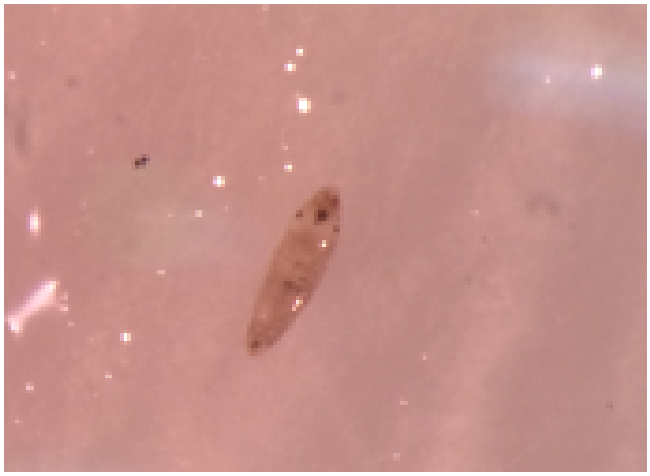

Figure 4. Determining the presence or absence of embryos. After bleaching the eggs. The egg chorion dissolves and leaves the embryos. These can be hard to see, but can be identified by their eye spots

Suggested schedule for the experiment:

SCHEDULE/Date	**LAB**
Strain:	*Aedes spp*
Tuesday	**Hatch**
Wednesday	rear
Thursday	rear
Friday	rear
Saturday	rear
Sunday	rear
Monday	Expect first pupation. Remove pupae in afternoon
Tuesday	**collect pupae in afternoon**
Wednesday	(sex and tube females)

Thursday	**Adults emerge**
Friday	Irradiate males and cage with 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

insects

MDPI

Article

Suitability of Raycell MK2 Blood X-ray Irradiator for the Use in the Sterile Insect Technique: Dose Response in Fruit Flies, Tsetse Flies and Mosquitoes

Hanano Yamada [1,†], Bénéwendé Aristide Kaboré [1,2,3,*,†], Nanwintoum Séverin Bimbilé Somda [1,4], Nonhlanhla L. Ntoyi [1,5], Chantel Janet de Beer [1], Jérémy Bouyer [1], Carlos Caceres [1], Robert L. Mach [2] and Yeudiel Gómez-Simuta [6]

1 Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna International Centre, 1400 Vienna, Austria
2 Institute of Chemical, Environmental and Bioscience Engineering, Vienna University of Technology, Gumpendorfer Straße 1a, 1060 Vienna, Austria
3 Insectarium de Bobo-Dioulasso-Campagne d'Eradication de la mouche Tsétsé et de la Trypanosomose, Bobo-Dioulasso BP 1087, Burkina Faso
4 Unité de Formation et de Recherche en Science et Technologie (UFR/ST), Université Norbert ZONGO (UNZ), Koudougou BP 376, Burkina Faso
5 Vector Reference Laboratory, National Health Laboratory Services, Centre for Emerging Zoonotic and Parasitic Diseases, National Institute for Communicable Diseases, Johannesburg 2192, South Africa
6 Programa Operativo Moscas, IICA-SENASICA Km 19.5, Carretera Tapachula-Ciudad Hidalgo, Metapa de Dominguez 30860, Chiapas, Mexico
* Correspondence: a.kabore@iaea.org or benarist.vet@gmail.com; Tel.: +43-688-64381248 or +226-70343535
† These authors contributed equally to this manuscript.

Simple Summary: The sterile insect technique (SIT) is an environment-friendly, species-specific pest control method by which target insects are mass-produced in a factory and are made infertile by irradiation—usually with gamma rays. However, gamma sources are becoming more difficult and expensive to purchase, and the regulations surrounding these types of irradiators are becoming stricter. Therefore, there is now increasing interest in alternatives, such as X-ray irradiators. Following a recent technical evaluation of a blood X-ray unit, the aim of this research was to assess the biological responses of a selection of major SIT target insect species to irradiation in the X-ray unit as compared to gamma ray irradiation. It was found that all the insects responded similarly to X-rays as to gamma rays and that the X-ray unit is suitable for small- to medium-sized SIT programs.

Abstract: The sterile insect technique (SIT) is based on the inundatory field release of a target pest following their reproductive sterilization via exposure to radiation. Until recently, gamma irradiation from isotopic sources has been the most widely used in SIT programs. As isotopic sources are becoming increasingly expensive, especially for small programs, and regulations surrounding their procurement and shipment increasingly strict, irradiation capacity is one of the limiting factors in smaller or newly developing SIT projects. For this reason, the possibility of using X-ray irradiators has been evaluated in the recent decade. The availability of "off-the-shelf" blood X-ray irradiators that meet the technical requirements for insect irradiation can provide irradiation capacity for those SIT projects in which the acquisition of gamma ray irradiators is not feasible. Following the recent technical characterization of a Raycell MK2 X-ray blood irradiator, it was found in this study, that MK2 instruments were suitable for the sterilization of fruit flies, tsetse flies and mosquitoes, inducing comparable, even slightly higher, sterility levels compared to those achieved by gamma ray irradiation. This, together with its estimated processing efficiency, shows that MK2 irradiators are suitable for small- to mid-sized SIT programs.

Keywords: *Ceratitis capitata*; *Anastrepha ludens*; *Glossina palpalis gambiensis*; *Aedes aegypti*; *Anopheles arabiensis*; X-ray; gamma ray; sterility; SIT

check for updates

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1. Introduction

Irradiation-induced sterilization of insects is an integral part of the sterile insect technique (SIT) [1] in which target pest species are produced in mass-rearing facilities, and males are made infertile before releasing them into a field site. Successful mating between the sterile males and wild females lead to a progressive decline in the target pest population density over successive generations and, thus, reduces crop loss and preserves animal, as well as human, health [1–3]. The Insect Pest Control Laboratory (IPCL) of the Joint FAO/IAEA Center of Nuclear Techniques in Food and Agriculture in Seibersdorf, Austria, houses several species and strains of fruit flies, tsetse flies and mosquitoes and has been driving research for the development of a SIT package against these insect pests of crops, livestock and human health. The most notable research toward the development or optimization of a SIT package against these pests in the past ten years was reviewed in [4].

The process of reproductive sterilization is one of the mandatory components of the SIT, and exposure to gamma radiation from isotopic sources is, to date, the most efficient, reliable and widespread method to achieve sterility in insects [5], especially in large SIT programs. However, the regulatory challenges and costs of procuring and transporting radioactive sources are very high and still rising, making the acquisition of 60Co-based irradiators a limiting factor for many SIT facilities, especially in the earlier phases of the projects [6]. The feasibility of using X-ray irradiators has been evaluated in the most recent decade, and it has been found that X-ray irradiation, in general, induces sterility in insects similarly to gamma ray irradiation in tested insects [7–13]. Furthermore, the availability of "off-the-shelf" blood X-ray irradiators with competitive purchasing costs that meet the technical requirements for insect irradiation can now provide irradiation capacity for those SIT projects in which the acquisition of gamma ray irradiators is not feasible. A recent technical characterization of a Raycell MK2 X-ray blood irradiator (Best Theratronics Ltd., Kanata, ON, Canada) showed that this irradiator provided a dose uniformity ratio of under 1.2, an average dose rate of 7.7 Gy/min, and 2 L of irradiation capacity [6], thereby meeting the FAO/IAEA-recommended minimum criteria for insect irradiation with X-rays [14]. Following this initial assessment, the current study aims to complete an evaluation of an MK2 instrument by providing biological dosimetry data in the form of dose response curves for select insect pest species. The Plant Pests, Livestock Pests and Human Disease Vector groups of the IPCL [4] perform three separate experiments to assess the suitability of MK2 irradiators for the sterilization of fruit flies, tsetse flies and mosquitoes, respectively. The experimental set-up reflects the insect species, strains and life stages, sample preparation and irradiation processes and doses used, as expected to be used in SIT programs.

2. Materials and Methods

2.1. Irradiation Set-Up

All samples of insects were irradiated at a standard reference point, which was the center of a 2 L irradiation canister provided with the MK2 instrument used, as well as according to the dose rates and dose distribution map determined and described in Gómez-Simuta et al. [6]. Fruit fly and tsetse fly pupae were irradiated in instant rice for improved absorbed dose homogeneity in the sample and sample canister, as instant rice presents a similar density as insect pupae and, thus, serves as appropriate dummy material. Dose rates were, thus, measured in rice, and dose times were calculated accordingly. Mosquito pupae density is closer to water, whereas that of adults is closer to air. Dose time was, therefore, calculated according to dose rates measured in water and air, respectively. Where insects were additionally irradiated with a gamma ray irradiator for direct comparison, either a Foss Model 812 gamma irradiator (Foss Therapy Services Inc., North Hollywood, CA, USA) or a Gammacell 220 irradiator (Nordion Ltd., Kanata, ON, Canada) was used. These had dose rates of 56 Gy/min and 74 Gy/min, respectively.

2.2. Dosimetry

To ensure the accuracy of the irradiation dose given in each radiation event, Gafchromic HD-V2 or MD-V3 dosimetry films (International Specialty Products, Wayne, NJ, USA) were packed in small (2 × 2 cm) paper envelopes, which were placed near each insect sample. Gafchromic HD-V2 dosimetric films were previously indicated to be appropriate for the dose response of X-ray and gamma ray irradiation [6]. A DoseReader 4 instrument (Radiation General Ltd., Budapest, Hungary) appropriate for Gafchromic™ film [15] was used to read the films 24 h after irradiation. The standard operating procedure for Gafchromic™ film dosimetry [15] was followed to determine the absorption dose for each radiation event. The calibration used had a global uncertainty of 4.29%.

2.3. Dose Response of Ceratitis capitata and Anastrepha ludens Pupae under Hypoxic Conditions

2.3.1. Strains and Rearing

The *Ceratitis capitata* VIENNA 8 genetic sexing strain (GSS) was developed at the IPCL [16]. This GSS was characterized by a pupal color mutation in which a wildtype copy of the markers was attached to the Y chromosome so that males expressed the wild phenotype (brown pupae), and females expressed the mutant phenotype (white pupae sensitive to temperature). Female embryos could be eliminated at the embryo stage by exposing the eggs to high temperature (34 °C) for 24 h [16]. The *Anastrepha ludens* GSS was developed in Mexico [17] and transferred to the IPCL in 2017. The males expressed a brown pupae phenotype, while the black pupae phenotype was expressed in females; then, the sexes could be separated at the pupal stage by using a pupal color-sorting machine.

The laboratory rearing conditions of the flies were 24 ± 1 °C, 60 ± 5% relative humidity (RH) and a photoperiod of 14 h light: 10 h dark. The adult flies were fed with a standard adult diet [18,19], which consisted of sugar and hydrolyzed yeast in a ratio of 3:1 and water ad libitum. The flies in this study were kept in 30 × 30 × 45 cm (length, width and height, respectively) cages that were covered with muslin cloth and had openings for experimental handling. The larvae of the flies were maintained on a carrot-powder-based diet. The pupae were separated according to the color of the pupa, as described above.

2.3.2. Irradiation Procedure and Assessment of Sterility

Ceratitis capitata: Batches of brown pupae (males) two days before emergence were placed in plastic bags one hour before irradiation to achieve hypoxia, as described by Schwarz et al. [20] and the FAO/IAEA/USDA product quality control manual for fruit flies [21]. Two samples were irradiated separately in a Foss Model 812 Gamma Irradiator and in a Raycell MK2 irradiator at doses of 80, 90, 100, 125 and 145 Gy. Samples of pupae undergoing the same handling without irradiation were kept as controls.

After irradiation, pupae were kept in Plexiglas cages for fly emergence. Twenty-four hours after emergence, for each replicate, 20 sterile males and 20 fertile females were placed into a 30 × 30 × 30 plexiglass cage for sexual maturation, mating and oviposition. During the peak of the oviposition period, a sample of eggs was counted, placed on a piece of cloth, transferred to a larval diet and placed in an incubator at 28 °C for egg hatching. After 48 h, the number of unhatched eggs was recorded, and the number of pupae collected at the end of the experiment was registered. Five repetitions were performed for each dose and both the gamma and X-ray treatments.

Anastrepha ludens: Samples of *A. ludens* were collected, prepared and irradiated as described above for *C. capitata*. Only the dose of 80 Gy that is used in operational programs for the induction of reproductive sterility in this species was assessed for both the gamma ray (Foss Model 812) and X-ray (MK2) treatments. After irradiation, the pupae were returned to the insectary, and the dose response was assessed as described above for *C. capitata*. Nine repetitions were performed for each irradiator.

2.4. Dose Response of Glossina palpalis gambiensis Pupae

2.4.1. Strain and Rearing

The *Glossina palpalis gambiensis* colony used in this assessment was established at the IPCL in 2009 from pupae derived from the Centre International de Recherche-Developpement sur l'Elevage en zone Subhumide (CIRDES) colony in Burkina Faso. Initially, the strain was colonized at Maisons-Alfort (France) in 1972 using pupae collected in Guinguette (Burkina Faso) and transferred to CIRDES in 1975 [22]. The last wild material introduced into the colony was collected at Mare aux Hippopotames in 1981. The colony, as well as the pupae and adults used in the assessment, were maintained at a constant temperature and relative humidity (RH) of 24 ± 0.5 °C and 75–80%, respectively, and under subdued and indirect illumination with a 12 h light: 12 h dark photoperiod [23,24]. The colony and experimental flies were fed three times per week on defibrinated bovine blood using an artificial membrane feeding system.

Pupae that were produced in the colony were collected daily and sex-sorted with a newly developed Infrared Pupae Sex Sorter (NIRPSS) at 23–24 days following larviposition. The NIRPSS was preconditioned with the following melanization parameters: T1 of 252, T2 of 0.10 and T3 of 10. The male pupae were selected from the same cohort of pupae classed as unmelanized when the unmelanized ratio (unmelanized pupae/total pupae sorted) was below 38%.

2.4.2. Irradiation Procedure and Assessment of Sterility

Depending on the replication, fifty to seventy-five male *G. p. gambiensis* pupae were placed in a 60 mm × 13 mm petri dish without filling it, and this was placed in the middle of a cylindrical sample canister (2.0 l, 167 mm (Ø), 97 mm(H)) accompanying the Raycell MK2 instrument used. The remaining volume of the sample canister was filled with rice. The pupae were then exposed to radiation doses of 70, 90, 110 and 130 Gy. The control group was selected from pupae that were not irradiated. All irradiated and control pupae were handled and kept under similar conditions.

The irradiated and control pupae were incubated at 24 ± 0.5 °C and 75–80% RH until emergence. The teneral males were collected and kept in small cages (110 mm (Ø); 45 mm (H)) and fed as described above until sexual maturity. The females that emerged, due to a sorting error, were discarded. The seven- to eight-day-old irradiated and control males were mated in standard colony cages (Ø 20 cm) with three- to four-day-old virgin females at a 1:1 or slightly below male (N = 431): female (N = 592) ratio for four days, and their mortality was monitored daily. Males and females were then separated by chilling at 4 °C. The females were transferred to 20 cm diameter cages, and their daily production and mortality rates were recorded for 60 days. Six replications were performed for all doses.

2.5. Dose Response of Aedes aegypti and Anopheles arabiensis Pupae and Adults

2.5.1. Strains and Rearing

The *Aedes aegypti* strain originated from field collections in Juazeiro (Bahia), Brazil, and was transferred to the ICPL from the insectary of Biofabrica Moscamed, Juazeiro, Brazil, in 2016. Both the *Aedes* strains were maintained following the "Guidelines for Routine Colony Maintenance of *Aedes* mosquitoes" [25]. The Dongola strain of *Anopheles arabiensis*, originating from Dongola, Northern State, Sudan, was donated by the Tropical Medical Research Institute, Khartoum, Sudan, in 2010 and was maintained at the IPCL following the anopheline mass-rearing guidelines [26].

2.5.2. Irradiation Procedure and Assessment of Sterility

Eggs of *Aedes aegypti* from one cohort were collected and split in half to be hatched two days apart (one batch for collecting adults and one for collecting pupae for irradiation simultaneously). Males that emerged within an 8 h window were collected, counted into batches of 30, and placed in 15 × 15 × 15 cm Bugdorm® cages (MegaView Science Co. Ltd., Taichung, Taiwan). The next day, the adult males were transferred to and irradiated in

small 2 cL plastic cups closed with sponges. At the time of irradiation, the adults were 24–32 h old.

Pupae from the same cohort were collected in 4 h windows to ensure a uniform pupal age of 40–44 h, which is the most radioresistant age in this species. The pupae were sexed based on pupal size using a glass pupal sorter [27], and sex was verified under a stereomicroscope. All males were kept for irradiation, and females were transferred to individual tubes for emergence to ensure virginity for later mating. Male pupae were batched into groups of 30 in 2 cL plastic cups with excess water removed for irradiation.

The irradiation doses were selected according to the expected dose required to induce 50–100% sterility: 20, 55, 70 and 90 Gy. Both the pupae and adults in each technical repetition were irradiated simultaneously in a Raycell MK2 irradiator. Six repetitions were performed for all doses. Controls received the same handling but were not irradiated.

Anopheles arabiensis pupae were collected and sexed visually using a stereomicroscope. Females were placed in individual tubes for emergence to ensure virginity and were kept for later mating. Male pupae were counted into batches of 30 and were placed inside 2 cL plastic cups with excess water removed for irradiation. Male adults were knocked down in a cold room, counted into batches of 30 and placed into plastic tubes for irradiation. At the time of irradiation, male pupae were 24–28 h old, and adults were 24–30 h old. Both pupae and adults were irradiated in a Raycell MK2 irradiator with doses of 75, 90, 100, 110 and 120 Gy. Controls received the same handling but were not irradiated. Additional sample batches including controls were collected, sexed and prepared for irradiation with the same procedures but were irradiated in a GC220 gamma ray irradiator, with 55, 70, 95 and 110 Gy.

Following irradiation of both species, the males of each treatment group were placed in separate 15 × 15 × 15 cm Bugdorm® cages. Thirty virgin females were added to each cage when the adults reached 2–3 days of age and were allowed to mate for 3 days before they were provided with 2 bloodmeals on consecutive days (days 6 and 7 after emergence). Oviposition cups were added to each cage on day 8 for mass egg collection (on days 9 and 10 after emergence) and were hatched following routine rearing protocols [25,26]. The total numbers of hatched and unhatched eggs were counted using a stereomicroscope. Any nonhatched eggs were either opened with a dissection needle, or if there were many, bleached to determine fertility status [28].

2.6. Statistical Analyses

The tsetse pupae emergence rate was analyzed using a generalized linear mixed model, where the dose was considered a fixed effect and the replicates as random effects. An emmeans comparison with the Tukey method was used to assess the differences between the irradiation dose treatments. The induced sterility of the tsetse flies was calculated by subtracting from 100% (pupae production in the control group) the treatment production relative to the control group, which was obtained by dividing the pupae produced in each irradiation dose treatment by the pupae produced in the control group.

Sterility in the fruit flies was calculated as the percentage egg hatch of the control group hatch rate. A Wilcoxon rank sum test with continuity correction was used to compare hatch rates of gamma- and X-ray-irradiated fruit flies. The residual fertility (RF) for mosquitoes was calculated as a proportion of the control fertility of each treatment group (RF = HRtx/HRc), where HRtx was the hatch rate of the treatment (tx) group, and HRc was the hatch rate of the control (c) group. Induced sterility (IS) was calculated by subtracting the RF from 1.

To analyze the dose response of pupae versus adults for *Ae. aegypti* and *An. Arabiensis*, a binomial GLMM fit by maximum likelihood (Laplace approximation) was used for egg hatch rates (considered as response variables), life stage (2 levels: pupae and adults) and irradiation log (dose) (4 levels: 20, 55, 70 and 90 Gy), and their interactions were considered fixed effects, with repetition as a random effect.

3. Results

3.1. Dosimetry

The dosimetry confirmed that all doses received lay within a 4.29% error range.

3.2. Sterilization Efficiency of Raycell MK2

All insect species used in this study responded to the X-ray irradiation in the MK2 instrument as expected, with induced sterility levels comparable to those achieved in alternative X-ray and gamma ray irradiators using the same doses. When comparing the dose responses in pupae of all five species, *Ae. aegypti* were the most radiosensitive, becoming nearly fully sterile (99.8% IS) at doses of 55 Gy and above. *A. ludens* were fully sterile at 80 Gy, whereas *An. arabiensis* and *C. capitata* showed similar dose response curves and needed at least 100 Gy to achieve above 99.9% IS. Finally, *G.p. gambiensis* needed a dose of 110 Gy or above to reach 99.6% IS (Figure 1).

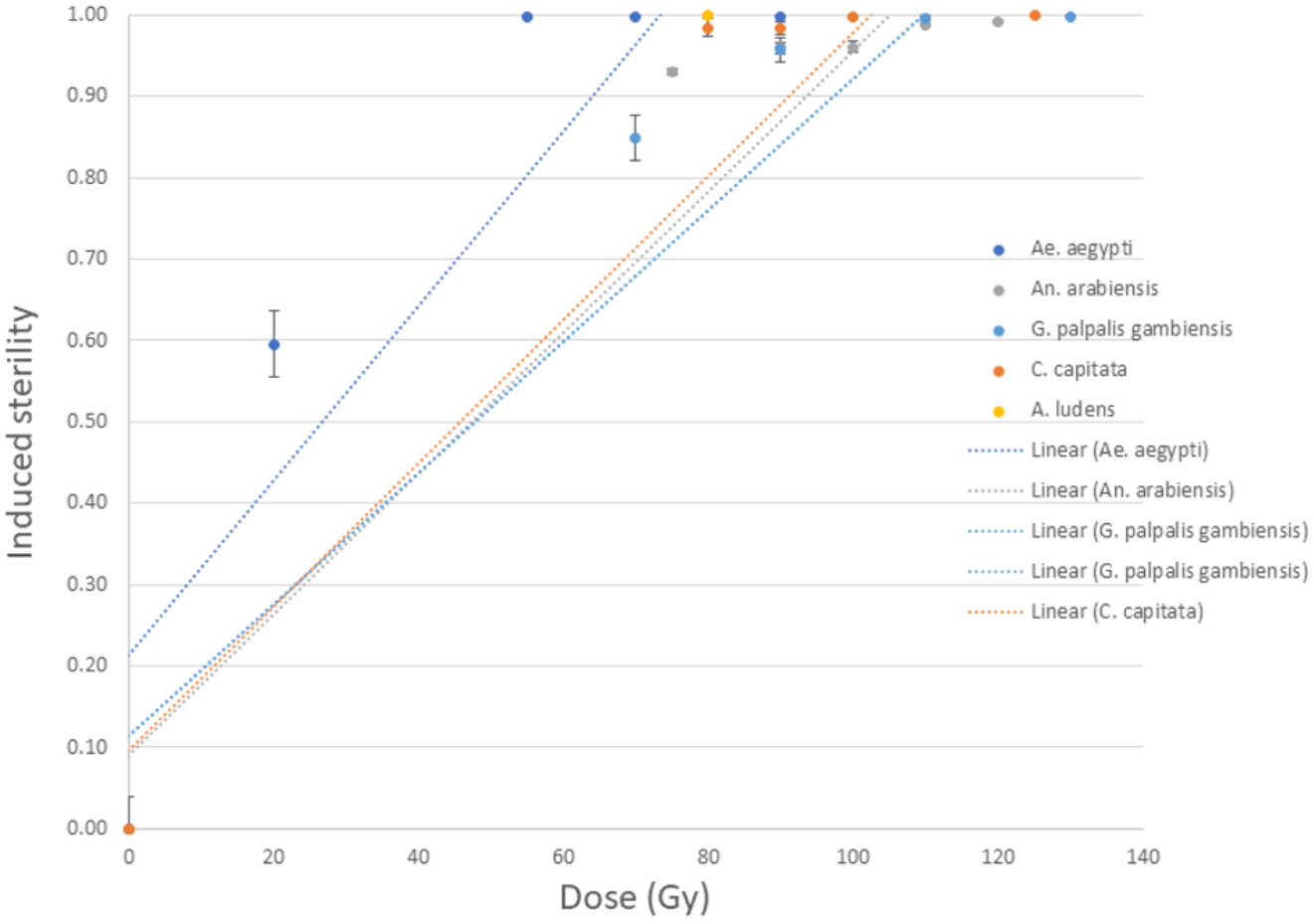

Figure 1. Induced sterility of pupae of five insect species in response to increasing irradiation doses in an MK2 irradiator. *A. ludens* was subjected to only one dose.

3.2.1. *Ceratitis capitata* and *Anastrepha ludens* Pupae

Ceratitis capitata pupae showed slightly higher levels of sterility (<2%) following irradiation with X-rays in the MK2 irradiator compared to irradiation with gamma rays (FOSS 812), (p = 0.036); a dose of 100 Gy resulted in 99.7% and 98.7% induced sterility (IS), respectively. Both the 125 Gy and 145 Gy doses gave full sterility, regardless of irradiator type. The dose responses following irradiation doses of 80, 90 and 100 Gy compared to the same doses of gamma irradiation are shown in Figure 2.

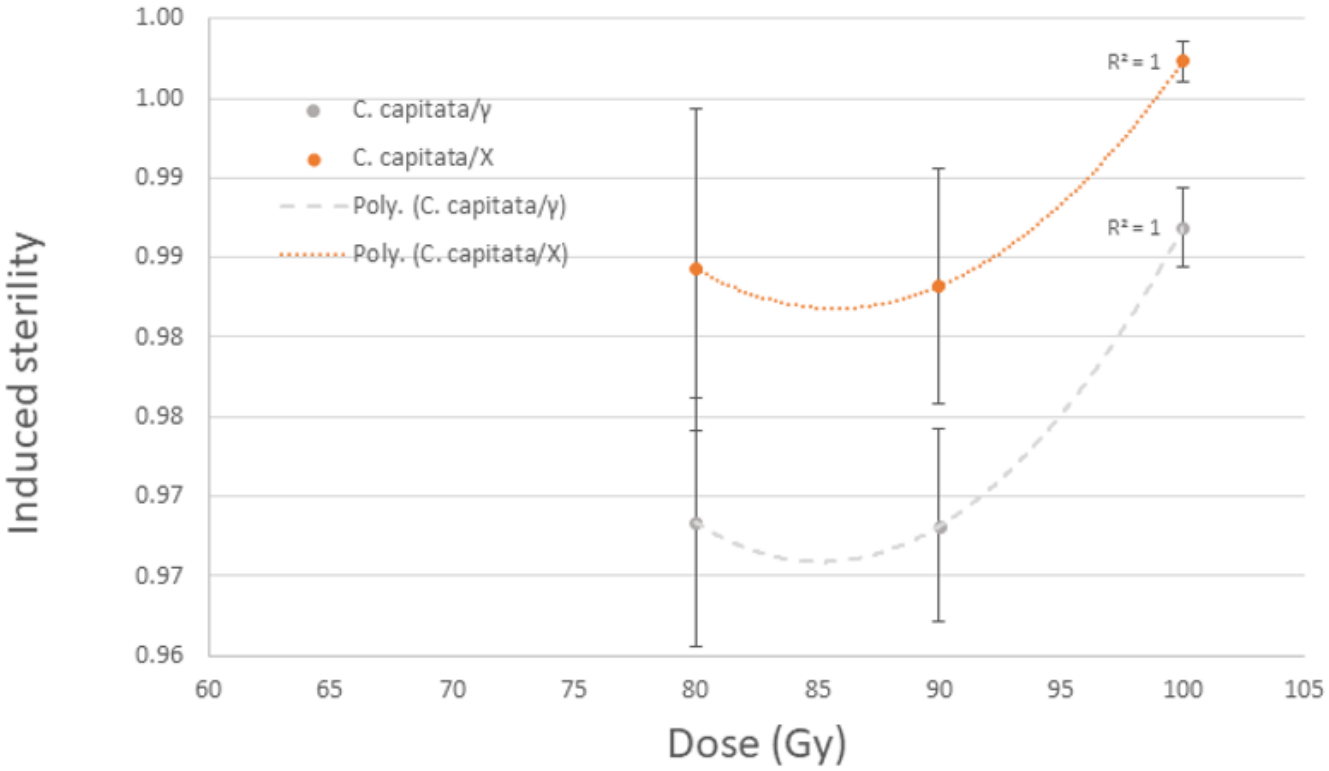

Figure 2. Dose responses of *C. capitata* following irradiation with 80, 90 and 100 Gy with X rays in an MK2 irradiator compared to γ-rays (Foss Model 812).

Anastrepha ludens irradiated as pupae in hypoxic conditions with 80 Gy with both X-rays (MK2) and gamma rays (FOSS 812) resulted in 100% sterility.

3.2.2. *Glossina palpalis gambiensis* Pupae

After exposure to radiation, the pupae were incubated until emergence. A decrease in emergence rate from 89.7% to 83.8% was observed as the dose increased. A significant difference in the emergence rate was observed (X2 = 14.332, df = 4, $p = 0.006$) and was higher in pupae irradiated with 90 Gy compared to those irradiated with 110 and 130 Gy. The total number of eggs aborted was higher in females mated with irradiated males than those mated with fertile males at all doses ($p < 0.001$), and this was inversely correlated to the pupae production. The number of eggs aborted by females mated with males irradiated at 90 Gy and 110 Gy was higher than the number in females mated with males irradiated with 70 Gy. The fecundity of females mated with irradiated males decreased from 0.012 to < 0.001 as the irradiation doses increased from 70 to 130 Gy. In contrast, the mean induced sterility in females mated with irradiated males increased from 84.9 ± 6.7 to 95.7 ± 3.6, 99.6 ± 0.6 and 99.8 ± 0.5% as the irradiation doses increased from 70 to 90, 110 and 130 Gy, respectively, when using the MK2 instrument (Figure 3).

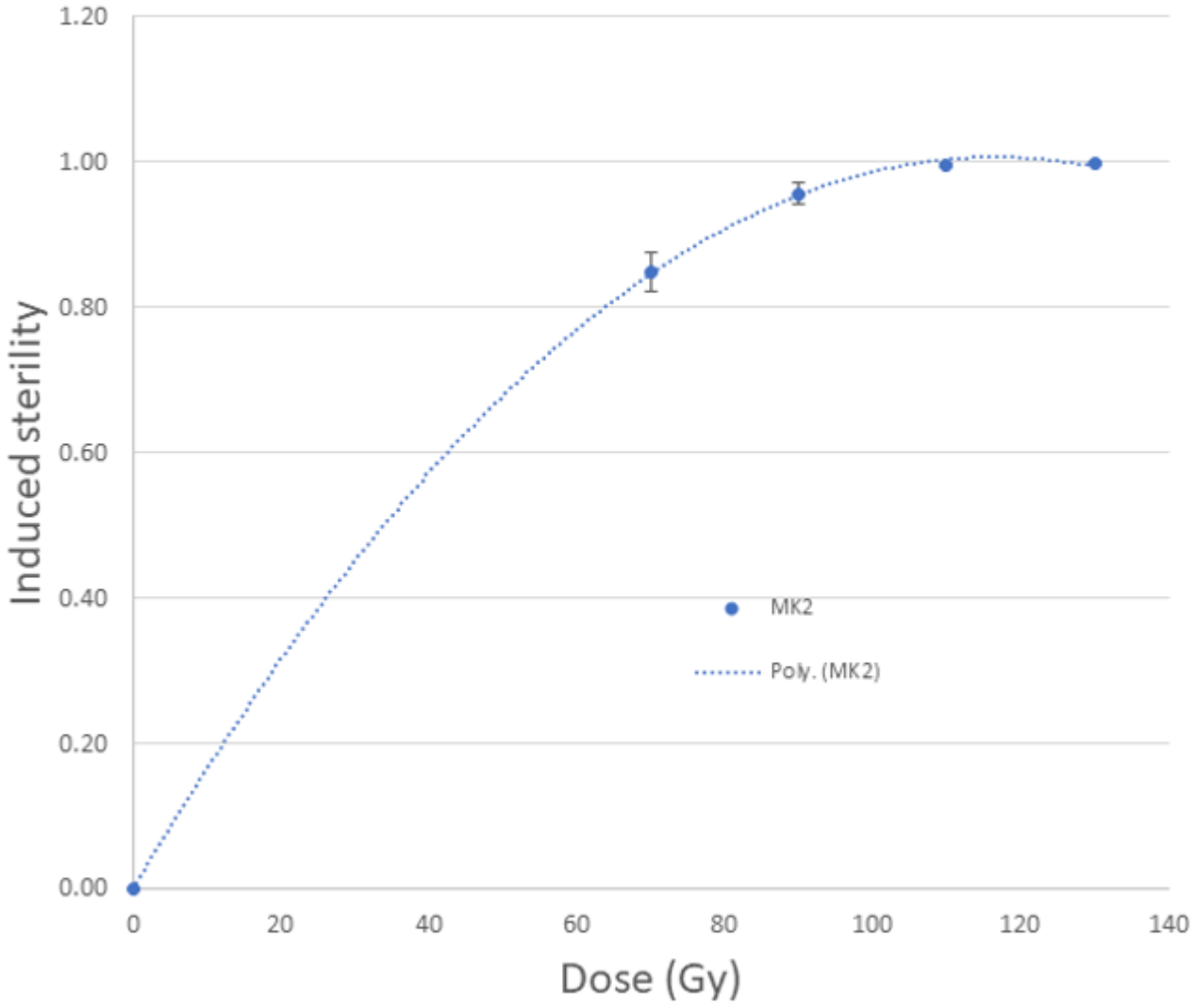

Figure 3. Dose response curve of *Glossina palpalis gambiensis* pupae irradiated with X-rays in an MK2 irradiator.

3.2.3. *Aedes aegypti* and *Anopheles arabiensis* Pupae and Adults

As expected, the hatch rates of both *Ae. aegypti* and *An. arabiensis* reduced significantly with increasing dose (df = 4, $p < 2.2 \times 10^{-16}$). When irradiated in the MK2 instrument, *Ae. aegypti* male pupae and adults presented with sterility levels exceeding those observed following irradiation with gamma rays (Figure 4). In these species, no difference in radiosensitivity between the two developmental stages could be observed following exposures in both irradiators, as a dose of 55 Gy or above led to very high sterility of over 99.9% (Figure 4), (df = 1; $p = 0.172$).

Both male adults and pupae of *An. arabiensis* responded to X-ray irradiation in the MK2 irradiator similarly to gamma ray irradiation. Induced sterility was, however, slightly higher following X-ray irradiation (Figure 5). The adult stage in this species was also more radiosensitive than the pupal stage (df = 1; $p = < 2.2 \times 10^{-16}$), which corroborates data for the same strain irradiated with gamma rays (GC220) (Figure 5).

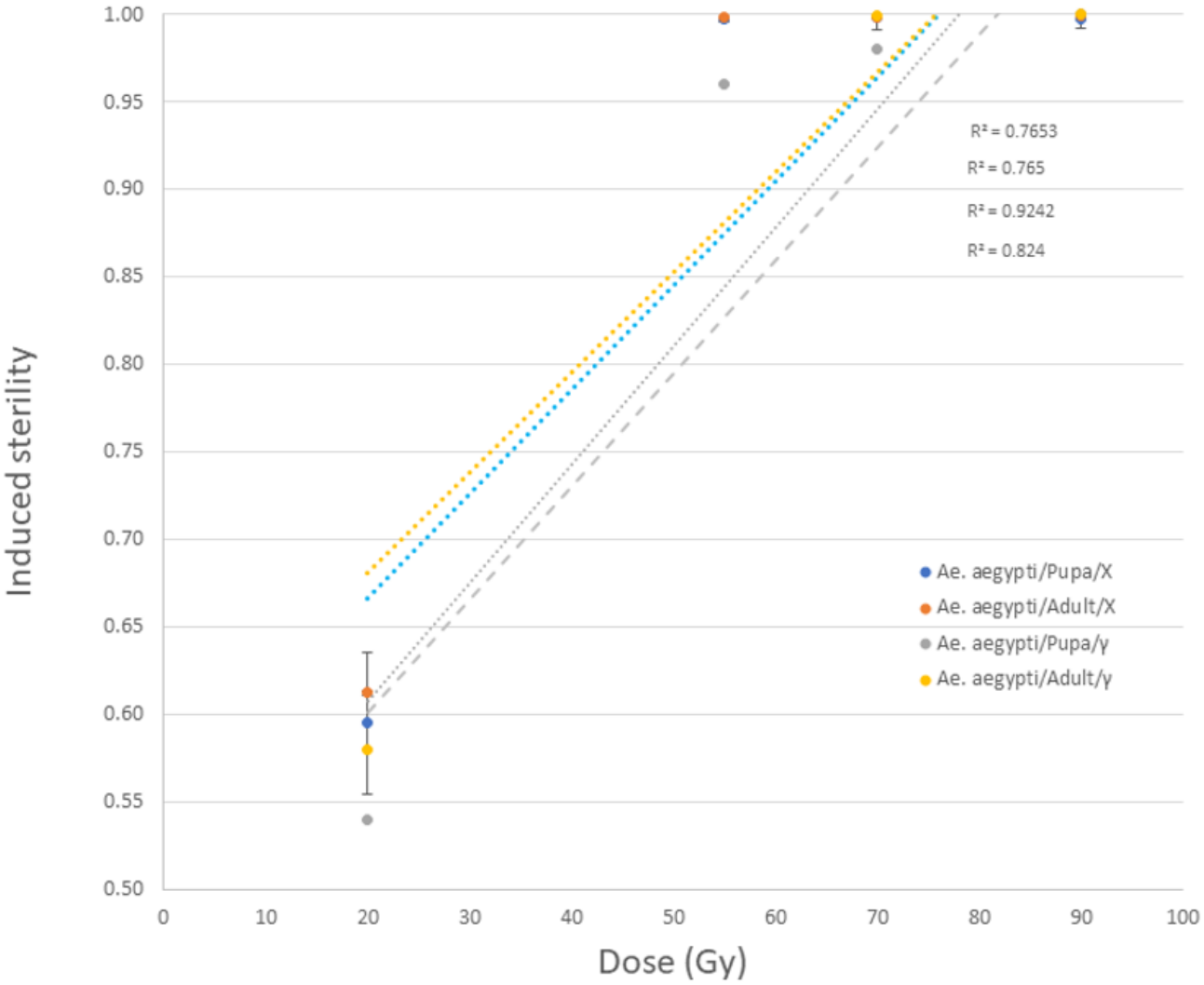

Figure 4. Dose response data of *Ae. aegypti* male pupae and adults irradiated with MK2 (X-ray) irradiator compared to the same strain irradiated in a GC220 instrument (gamma ray, (Yamada et al., 2022 [35])).

4. Discussion

The sterility data obtained from the five insect species tested in this report confirmed the relative biological effectiveness of MK2 irradiators compared to other X-ray and gamma ray irradiators. In the two tested fruit fly species, *C. capitata* was more sensitive following irradiation in the MK2 instrument than in the gamma ray irradiator, and *A. ludens* was fully sterile at the tested dose following irradiation in both irradiators. In a relevant study by Mastrangelo et al. [9], a (at the time) new generation of X-ray irradiator (RadSource2400) was evaluated in which the dose responses of *C. capitata* and *Anastrepha fraterculus* were assessed. It was also found that the exposure of the two fruit fly species to X-rays resulted in higher levels of sterility compared to gamma rays. In this case, 99% sterility in *C. capitata* was achieved with mean doses of 91.2 Gy with X-rays and 124.9 Gy with gamma rays, whereas 40–60 Gy was sufficient to sterilize *A. fraterculus* for both radiation treatments, which corroborates the results in this study. At present, most sterilization of insects is accomplished using gamma radiation, and considering that a dose of 80 Gy of gamma radiation was used in Anastrepha's mass-rearing laboratories in some countries (such as Mexico and Guatemala) [5], our result of 100% sterility in *A. ludens* was achieved with 80 Gy of X-rays, which supports the suggestion that the use of X-rays could be an alternative technology for SIT with regard to biological dosimetry.

The MK2 irradiator was equally successful at sterilizing *G. p. gambiensis* pupae. Doses of 90 and 110 Gy were sufficient to induce 95.7 and 99.6% sterility in females that mated with exposed males. In other biological dosimetry tests for this species, gamma irradiation has been predominantly used for both adult and pupae sterilization, and for both life stages, a dose of 110 Gy only has induced sterility levels of 93.4% in adults [29] and 89.7% in pupae [30]. It has been reported in other studies that pupae are more sensitive to radiation than adults. When *Glossina brevipalpis* were treated as adults, a dose of 40 Gy induced 93% sterility in females, and the same dose when applied to pupae induced a sterility of 97% [31]. This variation in sensitivity between life stages was also seen for the subspecies of *Glossina palpalis palpalis* [32], for which a dose of 120 Gy was needed for adults and a dose of 60 Gy for pupae to induce sterility of 95%. The low induced sterility for pupae exposure to a dose of 110 Gy recorded by Ilboudo et al. [30] might be because of an error in the dose, as their dosimetry indicated an absorbed dose of 81 Gy. As an X-ray dose of 90 Gy was sufficient to induce sterility of 95.7%, further assessments to verify and compare the dose response of this species using two X-ray and one gamma ray irradiator are in progress.

In the two tested mosquito species, similarly, a lower X-ray dose was needed to reach the same level of sterility when compared to gamma irradiation, corroborating historical data where gamma ray irradiation has been applied. For *An. arabiensis* (Dongola strain), 110 Gy were required for >99% sterility in adults and 120 for pupae using the same Gammacell 220 machine with a cobalt-60 source and a dose rate of 16 Gy/min at the time of the experiment [33]. The GC220 irradiator was then introduced in 2010. Yamada et al. [34] observed higher residual fertility (14% and between 4 and 7%) in pupae of the same strain following irradiation in the same GC220 instrument with dose rates of 93 Gy/min (in earlier repetitions) and 84 Gy/min (in later repetitions), respectively [34]. In this study, using the same GC220 irradiator with a dose rate of 74 Gy/min at the time of the experiment, 110 Gy was sufficient to fully sterilize adults. However, pupae showed 4.6% residual fertility at the same dose. The sterility achieved in the MK2 irradiator in this particular strain of *An. arabiensis* showed fertility data lower than but closest to the data of Helinski et al. in 2006 [33]. This was likely because the dose rates of the MK2 are lower (average of 7.7 Gy/min) and closer to the GC220′s 16 Gy/min in 2006 than the other higher dose rates used in subsequent studies [35].

The dose response results for *Ae. aegypti* confirm former reports that *Aedes* spp. are generally more radiosensitive than Anophelines. In this particular experiment, male adults and pupae showed the same responses to the irradiation doses. However, a previous study showed that adults could be slightly more radiosensitive than pupae, although usually not significantly so [36]. This was not evident with the doses used in the MK2 irradiator, as doses of 55 Gy and above resulted in nearly full sterility. Other biological dosimetry tests performed in this strain of *Ae. aegypti* using a different X-ray irradiator (RS2400) with a dose rate of 9.11 Gy/min gave a very similar response curve (Yamada, unpublished data), whereas irradiation with gamma rays at lower dose rates induced higher sterility levels [37], and inversely, those with higher dose rates resulted in lower sterility levels [34].

These results, combined with the comparison of X-ray and gamma ray irradiation of *C. capitata*, support the findings of Yamada et al. [35], where it was shown that dose-dependent dose rate effects altered the dose response in mosquitoes and, likely, in other insects. At high doses, the higher the dose rate, the higher the residual fertility (and the lower the induced sterility). These new findings support our hypothesis that the increased sterilization efficiency of the X-ray irradiators is due to a dose rate effect. However, it is important to also investigate the effects of energy independent of dose and dose rate on insect dose response.

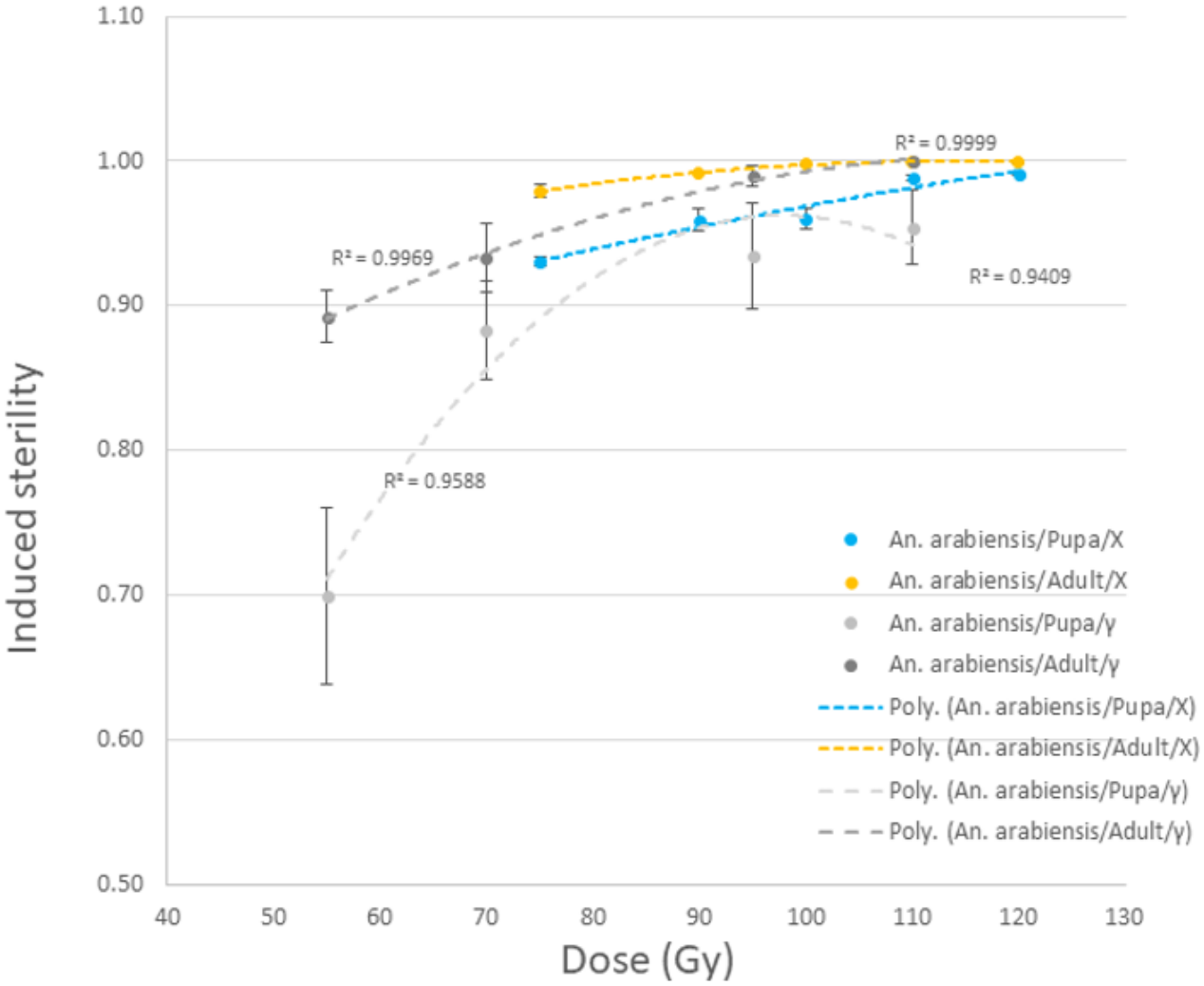

Figure 5. Dose response data of *An. arabiensis* male pupae and adults irradiated with MK2 (X-ray) irradiator compared to the same strain irradiated in GC220 irradiator (gamma ray).

Apart from the relative biological effectiveness of an irradiator, processing efficiency is important for the assessment of its suitability for operational SIT programs. The requirements for this depend on the size and production capacity of the program and, thus, can vary. The largest SIT programs currently are those controlling fruit flies; the highest-producing facility is the El Pino facility in Guatemala, which has a production capacity of 3.6 billion sterile males per week. For the sterilization of such quantities, high-dose-rate, high-capacity, self-shielded or panoramic irradiators are needed. Other programs that require smaller production numbers, such as those in Hawaii, Costa Rica, Australia and many pilot facilities, can be run adequately with smaller irradiators, such as self-shielded GC220 instruments or, alternatively, blood X-ray irradiators such as the MK2 machine. Using the full volume of the 2 L canister, a fruit fly SIT program can sterilize approximately 13.4 million *A. ludens* and 25 million *C. capitata* pupae per week with one 8 h shift per day, with the potential to increase the processing capacity to 26.9 and 50 million per week, respectively, with the implementation of a second shift per day. Of course, these numbers can be increased by procuring more than one X-ray unit.

The current protocol of pupae irradiation that is used for the SIT program against tsetse flies in Senegal indicates that the pupae are irradiated inside specialized boxes designed for pupae shipment [38]. The recommended density of pupae inside a box is 1500 [38–40], and four boxes can fit inside the 2 L canister (i.e., 6000 tsetse pupae). Thus, with a processing

time (exposure time for 110 Gy plus sample loading) of 19 min, three loads per hour can be irradiated. Therefore, around 1.1 million tsetse pupae can be treated in a 5-day week with two 6 h shifts. For the successful SIT program on Unguja Island (Zanzibar) in an area of 1650 km^2, the largest number of flies that were ever released in one week was 102,557 [41]. Thus, the processing capacity of the MK2 irradiator more than meets the requirements of similar-sized tsetse SIT programs. If the full capacity of the 2 L canister were to be used, it could hold around 48,000 tsetse pupae. The output could, thus, be increased to around 8.6 million tsetse sterile males each week. However, the handling and packing protocols would need adjusting so as to not damage the pupae during irradiation. Additionally, in SIT programs against tsetse flies (and mosquitoes), blood used for feeding the colonies requires sterilization with irradiation at 1 k Gy to minimize contamination with pathogens (https://www.iaea.org/sites/default/files/guidelines-for-blood-processing-procedures.pdf, accessed on 16 September 2022). This can also be accomplished in an MK2 irradiator in just over 2 h.

For mosquito SIT programs against *Aedes* spp., in theory, around 75 million adult mosquitoes can be treated in a 5-day week with two 6 h shifts, as the full volume of the 2 L canister can hold 250,000 Aedes adults, and a processing time (exposure time for ~60 Gy plus sample loading) of 12 min allows for five loads per hour. The processing capacity of *An. arabiensis* would be less, as double the dose and, thus, double the time (~20 min) is needed for the exposure itself, thus allowing for only three loads per hour. Additionally, this species is slightly larger, and fewer adults can be compacted into the same space [42]. These numbers are adequate for current pilot SIT trials, the largest of which releases around 10 million sterile *Ae. albopictus* males per week. However, it is anticipated that large-scale area-wide control programs may require much higher irradiation capacity, for which multiple self-contained X-ray irradiators would be needed if it is not possible to house an industrial, high-throughput irradiator. It is also important to note that adult mosquitoes need to be immobilized by, for example, chilling [35,42,43] to be compacted in a container and not sustain injuries when moving around while packed. The chilling needs to remain for the duration of the irradiation exposure if irradiation times exceed 4–5 min, by which time adults start to become active at room temperature.

The MK2 irradiator, in common with other X-ray sources, has several advantages over isotopic sources: lower capital cost, much lower transportation costs and much simpler regulation and access control. As the generation of X-rays relies on electrical power, the radiation can also be easily turned off by removing the power. Servicing is more straightforward, as there is no radiation to contend with, and replacement tubes can be supplied by regular carriers. The supply of replacement cobalt-60 sources is both expensive and problematical. There have been an increasing number of cases of denial or delay of shipments of radioactive material [44–48], and there are stringent regulations and rising costs. The lower energy of X-ray systems means that it is much easier to block radiation, and typical X-ray systems are self-shielded and do not require a special room to house them. Finally, the skills needed for the handling of high-level radioactive sources are scarce, whereas the skills for handling the high-voltage systems required for X-ray are available in most countries.

The downside is, if the electrical supply is not reliable, the system does not function. All X-ray systems require good cooling to prevent the tubes from overheating, which can be difficult in remote locations, and X-ray tubes are rather fragile and susceptible to damage during transport. X-ray dose rates are often much lower than those from isotopic irradiators, and the lower energy and, in some cases, beam configuration restrict the volume that can be irradiated. In addition, X-ray systems are more likely to suffer failures due to the fragility of the tubes and the complexity of the electronics, high-voltage systems and external cooling units.

X-ray systems, therefore, offer advantages to small SIT programs with their lower costs and simpler regulation but are not yet able to compete with isotopic irradiators for larger programs. Although some SIT programs have implemented e-beam technology for

high-throughput irradiation (for instance, 600 million *C. capitata* are irradiated per week in Spain), currently available industrial e-beam systems are very expensive, and purchase is not feasible for most facilities. Small, compact electron beam systems and flat panel X-ray technology show promise for the future but are not yet ready for use.

5. Conclusions

Overall, MK2 irradiators were suitable for the effective and reliable sterilization of three target insect groups of SIT. The observed biological responses to the X-ray irradiation were comparable to gamma ray irradiation—in this case, irradiation in an MK2 machine resulted in higher sterility levels than those obtained in the two tested gamma irradiators. Together with its good DUR and processing efficiency, the unit met the requirements for small- to medium-scale SIT programs for fruit flies, tsetse flies and mosquitoes. Further research on the effects of dose rate and energy can further the understanding of the differences between X-ray and gamma ray irradiation.

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