Increased biting rate and decreased *Wolbachia* density in irradiated mosquitoes: safety issues in the framework of genetic control strategies

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Abstract

Releasing considerable numbers of radiation-sterilized males is a promising strategy to suppress mosquito vectors. However, under large-scale settings, small percentages of biting females can also be released in the field.

Currently, the effects of the irradiation on host-seeking and host-biting behaviors have not been exhaustively investigated. Information is also lacking regarding the effects of a sterilizing treatment on the endosymbiotic bacterium *Wolbachia*, that is known to attenuate the vector competence of infected mosquito species.

To ascertain the risks associated with the release of irradiated females, the pupae of two *Aedes albopictus* strains, differing by their natural or artificial *Wolbachia* infection type, were X-ray-treated using various doses to monitor key fitness parameters and biting behavior over two weeks. The effect of the radiation on *Wolbachia* was investigated by qPCR and FISH analysis. Radiation doses of 35 and 45 Gy completely inhibited progeny production but did not significantly affect *Ae. albopictus* female survival and flight ability, and caused a tripling of the number of bites per female per week compared to untreated controls. These results were also confirmed in *Ae. aegypti* after treatment at 50 Gy. A partial *Ae. albopictus* female sterility was achieved at 28 Gy but the number of weekly bites more than doubled compared to the controls. The *Wolbachia* density showed a significant downward trend in 45-Gy-irradiated females and decreased with adult aging and in younger treated pupae. This trend was confirmed in ovaries but not in extraovarian tissues. FISH analysis evidenced a strongly reduced *Wolbachia*-specific fluorescence in the ovaries of 13±1-day-old females. These findings suggested that irradiation could markedly increase the vectorial capacity of female mosquitoes, raising concerns about open field programs involving the release of substantial numbers of irradiated mosquitoes in the absence of perfect sexing methods.

Author summary

To reduce the reproductive potential of a target wild population of mosquitoes, X- or gamma-ray-based irradiation is a common procedure used to induce sterility in males prior to their release in the field. A small percentage of females escaping the sexing procedure may be also unintentionally released. Herein, we tested the effect of the radiation treatment on *Aedes albopictus* females and found that doses commonly used in SIT programs were not sufficient to significantly reduce survival and flight abilities, and the treated females continued to seek hosts even after engorgement, tripling the weekly number of bites compared to the controls. This phenomenon was confirmed in irradiated *Ae. aegypti* females. Moreover, the radiation treatment reduced the density of the endosymbiotic *Wolbachia* bacteria and markedly affected their distribution in the tissues of *Ae. albopictus* possibly reducing their ability to affect the vector competence, which is common in infected *Aedes* species. This was more evident when radiation had been applied on younger pupae and it increased with female aging. Results suggest that large-scale control programs based on mosquito irradiation should include rigorous methods for perfect sexing to avoid the risk of release of a myriad enhanced vectors.

Introduction

Despite several attempts, human efforts devoted to eliminating vector-borne diseases rarely produce lasting results in terms of efficacy. Global warming is a major issue and vectors that rapidly adapt to a changing environment by developing resistance to pesticides and by acquiring useful selection mutations have demonstrated enhanced invasiveness [1–3].

Owing to their reproductive potential and capability to rapidly spread both by migration and passive transportation, mosquitoes are prominent vectors, and substantial investment and basic research efforts are necessary for the prevention and development of control measures.

Together with certain anopheline species, *Aedes* mosquitoes represent the main concern for human health as they are competent vectors of several arboviruses and the impressive spread of the diseases associated to these pathogens in the last decades verifies the inefficacy of the current control methods [4–6].

The negative effects associated with the extensive use of insecticides has caused researchers to shift their focus to development of innovative control strategies characterized by high specificity and eco-compatibility, and to target the reduction of the reproductive potential or the vector competence of mosquito populations. This objective may be pursued through the release of modified conspecific individuals carrying factors that induce sterility, lethality, or virus resistance by acting on the progeny produced from mating with wild types. Advanced biotechnological methods are being utilized based on genetic modification or the exploitation of useful microorganisms [7]. Methods based on genetic modification can be targeted to specific genes using various tools [8–10] but such approaches can be only applied in countries where the release of GMOs is legal.

Other methods are based on non-specific genetic modifications. Particularly, the Sterile Insect Technique (SIT) involves the use of opportune doses of mutagenic radiation to achieve the sterilization of laboratory-reared males, and utilizing such males as sterilizing tools for release *en masse* in the field [11, 12].

A similar result can be obtained via a natural phenomenon of egg inviability induced by the common endosymbiotic bacterium *Wolbachia* [13].

Insect males infected by certain strains of this bacterium are only reproductively compatible with females harboring the same *Wolbachia* strain, whereas the lack of the infection or infection with a non-compatible strain causes the production of inviable offspring. This natural post-mating reproductive barrier is known as Cytoplasmic Incompatibility (CI) and contributes to the spread of infected individuals in the wild, since infected females can produce viable progeny with both

infected and uninfected males (Unidirectional CI, Uni-CI) [14]. In species infected in nature by CI-inducing *Wolbachia* strains, a Bidirectional CI (Bi-CI) pattern can be established artificially by replacing the natural infection with a different incompatible strain of the bacterium, thereby obtaining an alternate infection type. In this case, males and females of both populations are reciprocally incompatible and the infection type is not expected to favor any population [15]. Both in the case of Uni- and Bi-CI, the possibility to transfer horizontally the bacterium between species allowed to exploit *Wolbachia* to produce incompatible males that can be released in a target area to reduce the reproductive potential of wild-type populations in the cases of both Uni- and Bi-CI patterns (Incompatible Insect Technique, IIT) [16–20].

Large-scale implementation of SIT and IIT must be combined with an efficient method of sexing since the presence of residual females involuntarily released with the males can result in the occurrence of temporary spikes in vector density. Furthermore, especially when *Wolbachia*-induced Uni-CI occurs, released females may be invasive, possibly leading to the undesired phenomena of population replacement [15, 21].

The implementation of SIT using mosquito strains capable of producing incompatible males has been suggested as a method to mitigate the unpredictable effects of female co-release. Mosquito females are more sensitive to radiation than males and this approach would result in utilizing CI to achieve full male sterilization, whereas the radiation dose may be lowered to levels that exert no effect on male fitness but are sufficient to induce the complete sterility of those females escaping the sexing procedures [22]. However, even if recent trials based on this approach have been proven successful in terms of local *Ae. albopictus* suppression, the strategy involves radiation doses typical of SIT alone to be applicable on a large scale, leading to a considerably reduced male mating competitiveness without avoiding the release of a few fertile females [23].

It should be noted that all studies aimed at implementing SIT against vector mosquitoes have been mainly focused on male radiation biology [12, 24]. However, the radiation doses needed to achieve female sterilization are known to remarkably damage the ovarian tissues [22] and previous studies have reported that oogenesis plays a role in the regulation of critical physiological processes such as the tendency to bite and blood-feed [25, 26], which are critical factors in determining the vectorial capacity of mosquitoes [27]. Therefore, an appropriate study on the effects of radiation on these physiological and behavioral traits is warranted.

A thorough investigation on the effects of irradiation on *Wolbachia* would also be necessary in the case of a SIT-IIT combined strategy. In fact, the bacterium *Wolbachia* has been shown to be affected by radiation [28, 29]; thus, this aspect should also be considered before conducting large-scale programs as the titer of this endosymbiont may be related to the vector competence of the host

[30]. Herein, we aimed to further the knowledge by determining whether irradiation interfered with host-biting and host-seeking behaviors in *Ae. albopictus* and *Ae. aegypti* to comprehensively evaluate the potential of application of SIT or SIT/IIT strategies in the absence of a perfect procedure for sexing. Furthermore, the effects of the radiation on the titer of *Wolbachia* in *Ae. albopictus* were evaluated in whole mosquito bodies by qPCR, applying the treatment at different pupal ages and analyzing the effects at two adult ages. Additionally, ovaries and extraovarian tissues were similarly studied. Finally, FISH analysis was also conducted on irradiated ovaries to visualize the effects of the treatment on the bacterial population and to acquire further useful information for the interpretation of the results. Two *Ae. albopictus* lines have been used in the experiments to determine whether different *Wolbachia* strains might affect the results. The resultant data will be useful for comparing IIT, SIT and SIT-IIT combined strategies in terms of safety and sustainability, and to more consciously evaluate their applicability on a large scale.

Materials and methods

Ae. albopictus and *Ae. aegypti* strains and rearing

Two *Ae. albopictus* strains and one *Ae. aegypti* strain were used in the experiments. S_{ANG} *Ae. albopictus* originated from wild-type individuals collected from Anguillara Sabazia (Rome) in 2006 and harbors *wAlbA* and *wAlbB* *Wolbachia*. AR_{wP} *Ae. albopictus* was established in 2008 through the transinfection of *Wolbachia*-cured S_{ANG} individuals with *wPip* *Wolbachia* from *Culex pipiens molestus* [31] and is characterized by a bidirectional incompatibility pattern with wild-type *Ae. albopictus* [32]. Both lines were reared under laboratory conditions at ENEA-Casaccia Research Center (Rome) and were periodically outcrossed with wild-type individuals from the same area to preserve the genetic variability [33]. The *Ae. aegypti* line was provided by the University of Camerino (Camerino, MC, Italy) where it had been laboratory-reared since 2006. Colony maintenance was conducted by raising larvae to adulthood inside 1-L larval trays at a density of 1 larva/mL, provided with liquid food according to the methods described by previous authors [33]. Adult mosquitoes were maintained inside 40 × 40 × 40 cm cages at $T = 28 \pm 1$ °C, $RH = 70\% \pm 10\%$, and L:D = 14:10 hours, and were supplied with water and 10% sucrose. Blood meals were provided via anesthetized mice in agreement with the Bioethics Committee for Animal Experimentation in Biomedical Research and following procedures approved by the ENEA

Bioethical Committee according to the EU directive 2010/63/EU. The mice belonged to a colony housed at CR ENEA Casaccia and maintained for experimentation based on the authorization N. 80/2017-PR released on February 2, 2017 by the Italian Ministry of Health. Blood feeding by female mosquitoes on human volunteers during the experiments was also approved by the ENEA Bioethical Committee, and only a few of the Authors (RM, EL, GL, and MC) were involved.

Radiation methods

Cohorts of *Ae. albopictus* and *Ae. aegypti* females belonging to the strains described above were irradiated with X-rays to enable their comparison with sham-exposed individuals (control). X-ray radiation was performed using the Gilardoni CHF 320G X-ray generator (Gilardoni S.p.A.; Mandello del Lario, Lecco, Italy) operated at 250 kVp, and 15 mA, with filters of 2.0 mm of Al and 0.5 mm of Cu, furnished by the Physical Technologies for Security and Health Division of ENEA. Depending on the experiment and according to doses already tested for the radiation-based sterilization of the two species [12, 22, 23], 28, 35, and 45 Gy (dose rate: 0.868 ± 0.004 Gy/min, mean $\pm$ SD) were applied to *Ae. albopictus* pupae, while a single dose of 50 Gy was used to treat *Ae. aegypti*, since this dosage is known to fully inhibit egg production in the species [12]. Groups of 100 female pupae were transferred into a Petri dish (d= 4 cm) at 36 ± 4 hours of age (unless specified differently) and then transported to the irradiation facility. Immediately before commencement of irradiation, most of the residual water was removed using a glass pipette; irradiated pupae were then transferred in a larger water container to facilitate complete development and allow for adult emergence inside the experimental cages. Sham-exposed pupae, that is pupae treated as the exposed pupae excepted for X-ray exposure, were used as controls.

Survival, fecundity and fertility in irradiated *Ae. albopictus* females

Females irradiated at 28, 35, and 45 Gy, and untreated counterparts belonging to S_{ANG} or AR_{WP} *Ae. albopictus* were allowed to emerge inside 30 × 30 × 30 cm plastic cages which were checked for the presence of males that escaped the sexing procedure [19]. The number of females in each cage was adjusted to constitute a cohort of 30 virgin individuals to which 30 untreated males, characterized by the same *Wolbachia* infection type, were added.

The above-mentioned cages were used to monitor survival, fecundity, and fertility in the same treatments during the two weeks of observation and for the feeding behavior studies described below. Five repetitions were conducted.

Mortality was recorded daily by removing dead individuals. During the same two weeks, egg collection was initiated three days after the first blood meal and collection was interrupted at the fifth day after the last meal to limit overlapping with the second gonotrophic cycle. Egg collection devices were replaced every three days to avoid uncontrolled egg hatch. Eggs were maintained under wet conditions for three days; they were then allowed to dry and were counted to determine the fecundity rate. Egg fertility was assessed by counting hatched eggs after immersion in a nutrient broth [34].

Feeding behavior in irradiated *Ae. albopictus* and *Ae. aegypti* females

The feeding behavior of the females was monitored for five days starting from the fifth day after the emergence by offering a daily blood meal. The same procedure was conducted from the 12th to the 16th day, thus allowing for the study of a second gonotrophic cycle. The number of engorged females was registered daily within 20 min of placement of the blood meal in the cage. Results obtained with the two populations of females treated at the three radiation doses were compared with control untreated females. Each of these eight treatments was repeated five times.

Although the present study was focused on *Ae. albopictus*, to generalize the experiment, *Ae. aegypti* females irradiated at 50 Gy were similarly tested (in triplicate) in comparison with untreated controls. The hatch rate of irradiated *Ae. aegypti* was also measured and compared to control females to ascertain a successful radiation treatment.

Host-seeking ability and biting rate in irradiated *Ae. albopictus* females under large enclosures

The host-seeking behavior of AR_{WP} and S_{ANG} females irradiated at 45 Gy was studied under large enclosure conditions in comparison with untreated individuals taking a cue from previous studies [35]. The trials were conducted in two large cellular polycarbonate experimental units (LEU, 8.5 × 5 × 5 m L : W : H) with two large lateral openings (L = 8 m; H = 1 m) protected by mosquito net to promote ventilation and ensure natural climatic conditions. The latter were constantly monitored by a CR-10 data logger (Campbell Scientific, Logan, UT, USA). LEUs contained benches with soil

and potted plants. For each experiment, one volunteer was considered as a source of blood (host) and a second experimenter (collector) released, recovered, and counted the *Ae. albopictus* females after they had fed on the host. Both researchers wore mosquito net hats. The host also wore a long-sleeved shirt and short pants, exposing only the lower legs to limit the area for the mosquitoes to land on, whereas the collector wore a white tracksuit and white shoes. Four groups consisting of 30 starved females aged 6 ± 1 days were compared, differing by their infection type (S_{ANG} or AR_{WP}) and treatment (irradiated and non-irradiated individuals). Females were released at a side of the LEU by removing the cover of the cage. The second experimenter then immediately reached the host at the opposite side of the LEU to collect data. The proportion of blood-fed females was registered together with the time taken by each individual to reach the host, at 30-s intervals. Females that landed on the host but did not bite were excluded from the count. The experiments lasted for a period of 15 min, and for each group, six repetitions were performed, taking care to alternate the experimental units and the host. The experiments were conducted in the late afternoon on sequential days during June 2020. According to the methods described above, laboratory-engorged females belonging to each group were also used to study the effect of the radiation treatment on the refractoriness at seeking a host 48 h after the first blood meal. After their release in the LEU, the same parameters studied in the first phase of the experiment were considered. At the end of each experiment, an electric mosquito swatter and a powerful aspirator were used to eliminate any mosquitoes remaining inside the LEU.

Quantitative PCR analysis of the *Wolbachia* titer in irradiated *Ae. albopictus* females

Considering the effects of *Wolbachia* on vector competence [36], a qPCR analysis was performed on the strains of this bacterium present in S_{ANG} and AR_{WP} females after irradiation at 45 Gy, a dose known to induce full female sterilization in the species [12], and results were compared with those of untreated counterparts. In SIT operational programs, mosquitoes are generally irradiated at the pupal stage at 24-48 h; however, it is known that pupal age is one of the critical factors that markedly affects the biological response to the radiation dose [37]. Therefore, the effects of the irradiation were investigated analyzing DNA extracts of whole bodies of 6 ± 1 -day-old females developed from pupae irradiated at three ages (26 ± 2 , 36 ± 2 and 46 ± 2 h). Furthermore, since the density of *Wolbachia* is known to vary with *Ae. albopictus* female age [38], the titer of the bacterium was measured in ovaries and de-ovarized bodies of 6 ± 1 - and 13 ± 1 -day-old females after a treatment at 45 Gy was conducted on pupae aged 36 ± 4 h to investigate the radiation

effects on the bacterium in the germ line and somatic tissues. Untreated females were used as control.

DNA was extracted from single individuals using the ZR Tissue & Insect DNA Kit MicroPrep (Zymo Research, Irvine, CA, USA), according to manufacturer's instructions. When necessary, females were preliminarily chilled on ice and dissected using phosphate-buffered saline (PBS) to allow for the isolation of the ovaries from the other tissues, excluding the individuals with ovaries that were not intact.

Real-time PCR was performed using the Roche LightCycler® 96 Instrument (Roche Molecular Systems, Inc., Rotkreuz, Switzerland). Each reaction was performed in triplicate with a 20-μL final volume containing 10 μL of the Luna® Universal qPCR Master Mix (New England BioLabs, Ipswich, MA, USA), 0.03 μL of 150 nM each primer, and 2 μL of purified DNA using the following amplification program: initial activation at 95 °C for 120 s, followed by 40 cycles at 95 °C for 15 s and 60 °C for 40 s. The presence of specific amplification products was verified using dissociation curves [38].

Strain-specific primers were used to amplify the *wsp* loci [39], namely, the *wAlbA-wsp* and *wAlbB-wsp* loci in the case of *S_{ANG}* females, and the *wPip-wsp* loci in the case of ARwP specimens. *Wsp* plasmid standards were used to generate a standard curve for absolute quantification [38]. The *Ae. albopictus* actin gene was used as a nuclear reference of whole body extracts and extracts obtained after the removal of the ovaries and was amplified with the primer pair actAlbqPCR [40]. Owing to the marked decrease in actin gene copies observed preliminarily in irradiated ovaries (S1 Fig.), the normalization of qPCR data related to these organs was performed using total DNA content as reference [41]. For this purpose, the Thermo Scientific NanoDrop 2000 spectrophotometer (Thermo Fisher, Waltham, MA, USA) was used.

FISH analysis of the ovaries in irradiated *Ae. albopictus* females

Based on the results of qPCR, fluorescent in situ-hybridization (FISH) analysis was conducted on the ovaries of 13±1-day-old irradiated and untreated females according to the protocol described by previous authors [42]. Two hundred nanograms of the *Wolbachia* specific 16S rRNA probe (W2: 5'-CTTCTGTGAGTACCGTCATTATC-3') was added in the hybridization buffer [43]. Tissues were placed on a slide containing a drop of the VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and visualized using the Nikon Eclipse E800 confocal microscope and NIS-Elements 4.0 software (Nikon, Tokyo, Japan). An aposymbiotic population of *Ae. albopictus* [31] was used as the negative control.

Data analysis

Results were expressed as mean $\pm$ SE, and the arcsin square root transformation was applied to analyze proportional data. The Levene's test and the Shapiro-Wilk test were performed to assess equality of variances and normality, respectively. Statistical analysis was performed using the software PASW statistics (PASW Statistics for Windows, Version 18.0. SPSS Inc., Chicago, IL, USA) with the level of significance set at $P < 0.05$.

The survival curves of the four different treatments for each *Wolbachia* infection type, were compared using the Kaplan-Meier method and the log-rank (Mantel-Cox) test. The Kruskal-Wallis H-test followed by the Conover-Iman test was used to compare fecundity and egg hatch data obtained between treatments within each infection type.

Repeated measures ANOVA was used to analyze the bite data obtained between treatments over each week. Cages were considered the experimental units and data have been expressed as the mean percentage of engorged females per day, adjusted for cage-specific mortality. If Mauchly's test indicated a violation of the sphericity assumption, the degrees of freedom were corrected by using Huynh-Feldt estimates. Multiple comparisons between treatments were assessed using Tukey's HSD post hoc test. Additionally, one-way ANOVA was performed to compare the mean number of bites per female per treatment during each week of study.

Data regarding the host-seeking behavior under large enclosures were analyzed by assigning a value to the mosquitoes a value based on the median time of each catching interval to compute the average landing time. The proportion of engorged mosquitoes was measured considering the ones retrieved within the defined 15-min interval. Two-way ANOVA was used to analyze the differences between groups in terms of the proportions of females that had bitten the host and to compare average landing times. The Shapiro-Wilk test was conducted to ascertain that the proportions and average landing times were normally distributed.

One-way ANOVA was used to compare, within each *Wolbachia* strain, the qPCR data obtained from *Ae. albopictus* females treated at the three tested pupal ages or untreated. The effect of female aging on the overall titer of *Wolbachia* was also analyzed by performing one-way ANOVA within data of each strain of the bacterium. Data regarding ovaries and extraovarian tissues of the treated or untreated counterparts were similarly analyzed at the two tested female ages. In the case of rejection of the assumptions of equality of variance and/or normality, the Kruskal-Wallis rank-sum test was performed.

Results

Female survival, fecundity, and fertility in irradiated *Ae. albopictus* females

Regardless of the infection type, irradiation slightly reduced female survival but this difference was not statistically significant between treatments during the two weeks of observation (Fig 1; Log rank test for S_{ANG} : $\chi^2 = 2.43$; $df = 3$; $P = 0.49$; Log rank test for ARwP: $\chi^2 = 1.847$; $df = 3$; $P = 0.61$). A 45-Gy dose usage completely inhibited egg laying in *Ae. albopictus*, whereas few eggs were oviposited by females irradiated at 35 Gy. Among the latter, fertile ones were very rare (Table 1). A dose of 28 Gy induced considerable reduction in the number of oviposited eggs compared to untreated controls and markedly affected egg fertility, but did not fully impede progeny production (Table 1). These results were confirmed in both *Ae. albopictus* strains and both gonotrophic cycles.

Table 1. Fecundity and fertility of ARwP and S_{ANG} *Ae. albopictus* females irradiated at 28, 35 and 45 Gy compared to untreated counterparts over two gonotrophic cycles. Fecundity and egg hatching data related to *Ae. aegypti* irradiated at 50 Gy are also reported.

Mosquito line	Blood meal	Treatment	Mean ($\pm$ SE) eggs per female	Mean ($\pm$ SE) egg hatch (%) from the laid eggs
ARwP	1st	untreated	31.54 $\pm$ 0.77 a	85.87 $\pm$ 2.37 a
		28 Gy	5.67 $\pm$ 0.36 b	13.72 $\pm$ 1.77 b
		35 Gy	0.11 $\pm$ 0.04 c	0.00 $\pm$ 0.00 c
		45 Gy	0.00 $\pm$ 0.00 d	0.00 $\pm$ 0.00 c
	2nd	untreated	30.89 $\pm$ 0.79 a	85.61 $\pm$ 2.28 a
		28 Gy	5.04 $\pm$ 0.46 b	15.03 $\pm$ 1.91 b
		35 Gy	0.15 $\pm$ 0.04 c	0.00 $\pm$ 0.00 c
		45 Gy	0.00 $\pm$ 0.00 d	0.00 $\pm$ 0.00 c
S_{ANG}	1st	untreated	32.24 $\pm$ 0.82 a	87.44 $\pm$ 2.38 a
		28 Gy	5.45 $\pm$ 0.44 b	14.75 $\pm$ 2.28 b
		35 Gy	0.13 $\pm$ 0.04 c	0.00 $\pm$ 0.00 c
		45 Gy	0.00 $\pm$ 0.00 d	0.00 $\pm$ 0.00 c
	2nd	untreated	31.44 $\pm$ 0.79 a	86.77 $\pm$ 2.40 a
		28 Gy	5.27 $\pm$ 0.38 b	15.25 $\pm$ 1.60 b
		35 Gy	0.13 $\pm$ 0.03 c	2.86 $\pm$ 2.86 c
		45 Gy	0.00 $\pm$ 0.00 d	0.00 $\pm$ 0.00 d
<i>Ae. aegypti</i>	1st	50 Gy	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
	2nd	50 Gy	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00

ARwP = wPip *Wolbachia* infected *Ae. albopictus*;

S_{ANG} = wild-type *Ae. albopictus*;

Within each group and column, values followed by a different letter are significantly different by Kruskal-Wallis test followed by the Conover-Iman test ($P < 0.05$)

384

385 **Altered feeding behavior in irradiated *Ae. albopictus* and *Ae. aegypti* females**

386

387 Repeated measures ANOVA showed significant differences between treatments with regard to the
388 weekly biting activity (Fig. 2A; S_{ANG} -1st wk: Huynh-Feldt correction, $F_{(10.19, 54.35)} = 2.99$, $P < 0.05$;
389 S_{ANG} -2nd wk: Sphericity assumed, $F_{(12, 64)} = 4.52$, $P < 0.05$; AR_{WP} -1st wk: Sphericity assumed, $F_{(12, 64)} = 5.90$, $P < 0.05$;
390 AR_{WP} -2st wk: Huynh-Feldt correction, $F_{(9.12, 48.64)} = 3.36$, $P < 0.05$). Regardless
391 of the week and the infection type, untreated mosquitoes generally showed most remarkable biting
392 activity during the first day of blood meal availability and, then they generally rested while their
393 eggs underwent maturation. In contrast, irradiated females exhibited an anomalous blood feeding
394 behavior and the percentage of the daily engorged females out the total ones was subjected to just a
395 slight decrease over each week (Fig. 2A).

396 For the 35- and 45-Gy treatments, total bites per female approximately tripled weekly over those
397 recorded for the untreated females (Fig. 2B). During the first week, the mean number of bites per
398 S_{ANG} female averaged at 1.15 ± 0.07 , 2.14 ± 0.06 , 2.69 ± 0.07 , and 2.77 ± 0.07 when females were
399 untreated or had been treated, respectively, at 28, 35 and 45 Gy. Tukey's test evidenced significant
400 differences among untreated and treated females, and among females treated at 28 Gy and the other
401 three groups ($F_{3,16} = 118.01$; $P < 0.05$). During the second week, the above-mentioned values were,
402 respectively, 1.07 ± 0.05 , 1.89 ± 0.05 , 2.63 ± 0.03 , and 2.84 ± 0.06 and data of all treatments
403 differed significantly from each other ($F_{3,16} = 282.23$; $P < 0.05$) (Fig. 2B). With regard to the AR_{WP}
404 line, listing the groups in the same order as that mentioned above, the mean number of bites per
405 female were, respectively, 1.10 ± 0.03 , 2.15 ± 0.02 , 2.68 ± 0.03 , and 2.84 ± 0.02 , during the first
406 week ($F_{3,16} = 371.96$; $P < 0.05$), with the Tukey's test indicating significant differences between all
407 treatments; values of 1.05 ± 0.03 , 2.12 ± 0.02 , 2.70 ± 0.03 , and 2.76 ± 0.04 , during the second
408 week, were obtained ($F_{3,16} = 642.89$; $P < 0.05$), with significant differences observed between all
409 treatments except those at 35 and 45 Gy (Fig. 2B).

410 Similarly, 50 Gy-irradiated *Ae. aegypti* females evidenced an enhanced propensity to bite compared
411 to the untreated mosquitoes during both weeks of observation (Fig. 2C; 1st wk: Sphericity assumed,
412 $F_{(4, 16)} = 8.38$, $P < 0.05$; 2nd wk: Sphericity assumed, $F_{(4, 16)} = 11.51$, $P < 0.05$). Overall, the mean
413 number of bites for 50 Gy-treated females almost tripled that of untreated mosquitoes during the
414 first week (2.81 ± 0.03 and 1.03 ± 0.02 ; $F_{1,4} = 3315.67$; $P < 0.05$) as well as second week (2.86 ± 0.04
415 and 1.05 ± 0.03 ; $F_{1,4} = 1522.09$; $P < 0.05$) (Fig. 2D). The applied radiation dose was found to
416 induce complete sterility in *Ae. aegypti* females (Table 1).

417

Host-seeking behavior of irradiated *Ae. albopictus* females in large enclosures

The test was conducted at a mean temperature of 34.0 ± 1.0 °C and RH averaging $50.0\% \pm 5.0\%$ were registered.

The host-seeking behavior exhibited by irradiated females under large enclosure conditions reinforced the results obtained in small cages. The applied X-ray dose was not sufficient to reduce the ability of *Ae. albopictus* to reach the host in the LEU compared to untreated females (Fig.3A, B). The average landing times were not significantly affected by the infection type (two-way ANOVA: $F_{1,20} = 0.66$, $P = 0.43$), nor were they affected by the treatment (two-way ANOVA: $F_{1,20} = 0.93$, $P = 0.35$) in starved females. On average, landing times ($\pm$ SE) were, respectively, 346.31 ± 15.98 and 354.30 ± 10.74 in irradiated and untreated S_{ANG} females and 331.11 ± 12.74 and 348.29 ± 12.30 in irradiated and control AR_{WP} individuals.

In contrast, the biting behavior was remarkably affected by irradiation. Starved females did not reveal a significant difference in the proportion of biting females between untreated and 45 Gy-treated females (two-way ANOVA: $F_{1,20} = 0.59$, $P = 0.45$) or between infection types (two-way ANOVA: $F_{1,20} = 0.80$, $P = 0.38$) (Fig. 3A). Instead, the proportion of engorged females that repeated the blood meal was significantly higher in irradiated than in untreated mosquitoes (two-way ANOVA: $F_{1,20} = 276.53$, $P < 0.05$), while the infection type showed no remarkable effects (two-way ANOVA: $F_{1,20} = 1.13$, $P = 0.30$) (Fig. 3B). Consequently, a comparison between average landing times related to the engorged females was not performed due to the extremely reduced number of biting individuals among the untreated controls (0 in certain repetitions). To verify this result in older females that might have experienced the occurrence of age-related damages due to irradiation, the test was also repeated in triplicate with treated and untreated individuals aged 13 ± 1 days and similar results were obtained (S3 Fig.).

Decreased titer of *Wolbachia* in irradiated *Ae. albopictus* females

The age at which *Ae. albopictus* pupae had been irradiated at 45 Gy was found to affect significantly the density of these bacteria (*wAlbA*: $F_{(3, 36)}=2.96$, $P < 0.05$; *wAlbB*: $F_{(3, 36)}=4.05$, $P < 0.05$; *wPip*: $F_{(3, 36)}=6.76$, $P < 0.05$) regardless of the *Wolbachia* infection type. An early irradiation (26±2-hour-old pupae) induced a significant decrease in the whole body *Wolbachia* titer approximating a third in *wAlbA*, and a half in *wAlbB* and *wPip* strains (Fig. 4A) compared to the levels observed in the controls. As evidenced by the pairwise comparisons, the *Wolbachia* titer between treated and untreated individuals did not significantly differ when irradiation was performed on older pupae (36 and 46±2 hours old).

Adult female aging was also found to affect the titer of all the tested *Wolbachia* strains when a 45-Gy radiation treatment was performed (Fig. 4B). The difference between treatments was not statistically significant when the analysis had been performed on 6±1-day-old females (one-way ANOVA; *wAlbA*: $F_{(1, 18)}=1.54$, $P = 0.23$; *wAlbB*: $F_{(1, 18)}=0.82$, $P = 0.89$; *wPip*: $F_{(1, 18)}=3.28$, $P = 0.09$), but the decrease increased significantly in females aged 13±1 days (one-way ANOVA; *wAlbA*: $F_{(1, 18)}=62.11$, $P < 0.05$; *wAlbB*: $F_{(1, 18)}=38.93$, $P < 0.05$; *wPip*: $F_{(1, 18)}=4.60$, $P < 0.05$).

In agreement with the data obtained from the analysis of whole bodies, results of qPCR analysis also evidenced a correlation between aging and a reduced titer of the bacteria in the ovaries of irradiated compared to untreated *Ae. albopictus* females (Fig. 5A). This phenomenon was prominent when conducting analysis of females at 13±1 days after the irradiation (one-way ANOVA; *wAlbA*: $F_{(1, 18)}=62.11$, $P < 0.05$; *wAlbB*: $F_{(1, 18)}=5.91$, $P < 0.05$; *wPip*: $F_{(1, 18)}=4.60$, $P < 0.05$), whereas the *Wolbachia* density did not differ between treatments in 6±1-day-old females with regard to *wAlbA* and *wAlbB* *Wolbachia* strains (one-way ANOVA; *wAlbA*: $F_{(1, 18)}=0.39$, $P = 0.54$; *wAlbB*: $F_{(1, 18)}=0.50$, $P = 0.49$). A significant difference was found between the *wPip* *Wolbachia* titer in treated and untreated 6±1-day-old AR*wP* females ($F_{(1, 18)}=5.25$, $P < 0.05$). As also shown in the Figure 5A, the titer of *wAlbA* and *wAlbB* *Wolbachia* increased with aging in the ovaries of control females and confirmed previous studies [38], while irradiation led to an inversion of this trend. Regarding *wPip*, the controls evidenced an age-dependent decrease in titer and irradiation further intensified this trend in the density of the bacteria.

When testing the carcasses of the females without ovaries, the qPCR amplification results of *Wolbachia* (somatic fraction) did not reveal differences between treatments related to the titers of

wAlbA, *wAlbB* and *wPip* *Wolbachia* when irradiated females had been tested at 6 ± 1 days (Fig. 5B) (Kruskal Wallis test; *wAlbA*: $\chi^2 = 1.85$, $df = 1$, $P = 0.17$; *wAlbB*: $\chi^2 = 0.32$, $df = 1$; $P = 0.57$; *wPip*: $\chi^2 = 1.37$, $df = 1$, $P = 0.24$). In contrast with the results obtained in the ovaries, the titer of *Wolbachia* in the extraovarian tissues seemed to increase with aging in irradiated females compared to the controls but differences between treatments were not statistically significant (Kruskal Wallis test; *wAlbA*: $\chi^2 = 2.17$, $df = 1$, $P = 0.14$; *wAlbB*: $\chi^2 = 2.52$, $df = 1$, $P = 0.11$; *wPip*: $\chi^2 = 0.24$, $df = 1$, $P = 0.62$).

FISH analysis results

Quantitative PCR results related to the investigation of the ovaries were consistent with the images obtained by FISH analysis that showed occurrence of evident histological damage due to radiation with consequent reduction in the mean size of the organs (S3 Fig.), coupled with a markedly reduced *Wolbachia*-specific fluorescence intensity (Fig. 6, S4 Fig., S5 Fig., S6 Fig.). The analysis of the *Wolbachia*-infected individuals showed that fluorescent signals differed markedly between treatments (Fig. 6). In most of the ovaries dissected from *S_{ANG}* and *AR_{wP}* females irradiated at 45 Gy, a weak fluorescent signal was observed (Fig. 6B, 6D, S5 Fig., S6 Fig.) compared to untreated controls (Fig. 6A, 6C, S7 Fig., S8 Fig.). In certain cases, the fluorescence was only localized in the ovarioles that possibly had not been degenerated by the irradiation (Fig. 6D, red circle). In contrast, the organs maintained their authentic ovariole structure in untreated samples and the signal was abundant, specific, and confined within each oocyte (Fig. 6, S7 Fig., S8 Fig.).

Discussion

Several area-wide methods for mosquito control are under investigation based on the release of conspecific male individuals capable of reducing the reproductive potential of the wild-type populations through various mechanisms [7]. All these methods target specificity, efficacy, safety, and sustainability, in contrast to the traditional chemical-based methods and have demonstrated specific positive and negative aspects. Common to these strategies is the need to curb the release of a significant proportion of females and to prevent issues in the vector control strategies, failure to do which may potentially increase the load of vectors and the number of modified individuals capable of reproduction in the field. This work highlights how application of superior methods for sexing before release may be fundamental when males are sterilized through the application of radiation treatments.

Multiple blood-feeding is known to occur in *Ae. albopictus* as not all of the ovarian follicles exhibit commencement of the gonotrophic cycle with a single blood meal [44]. Studies conducted on engorged *Ae. aegypti* females demonstrated that they were generally inhibited from host-seeking by the occurrence of distension-induced and oocyte-induced mechanisms of regulation [25, 45]; however, this inhibition does not regard all individuals [44]. Results presented here demonstrate that the radiation doses characterizing SIT programs attenuate significantly this inhibition in both *Ae. albopictus* and *Ae. aegypti*, and that only the distension-induced mechanism of control may remain at least partially active. In fact, most females irradiated at 35 and 45 Gy gorged several times a week, even if the highest number of bites was generally observed on the first day of blood meal availability. Additionally, the dose of 28 Gy was found to be sufficient to induce a markedly enhanced biting behavior compared to the untreated controls. The tissue alterations experienced by the ovaries due to irradiation might explain this phenomenon as the host-seeking behavior is interrupted through the release in the hemocoel of hormones produced by the ovaries with oogenesis [46, 47]. In virus-endemic areas, multiple blood feeding indicates the existence of multiple occasions for a female mosquito to be infected by a suitable arbovirus and this possibility may remarkably increase the chances of virus transmission to other hosts. Furthermore, the comparison of the survival curves of irradiated and untreated females revealed that the tested irradiation doses were incapable of inducing a marked decrease in life expectancy within the first 20 days in *Ae. albopictus*, which is an age that only a minority of adults are expected to exceed in nature [48]. This evidence implies that irradiated *Ae. albopictus* females escaping the sexing procedure may have a stronger vectorial capacity compared to wild-type females, if fitness is sufficiently preserved. Previously, flight ability in radiation-sterilized males was not reported to be

affected when compared to untreated males [12]. The experiments testing the host-seeking ability under large enclosure conditions reported herein confirm that this biological feature is not affected by radiation doses that are capable of fully compromising fecundity in females. Indeed, starved females irradiated at 45 Gy did not exhibit a significantly different ability to reach and bite the host compared to the controls, but when engorged, their behavior changed and they continued to seek hosts, unlike the untreated females, leading to a remarkable increase in the mean number of bites per individual.

Overall, dose response curves related to fecundity and fertility showed agreement with the results of other studies [12] and verified the success of the radiation treatment. However, a dose of 28 Gy was incapable of inducing full sterilization of the produced eggs as was reported by previous authors [22]. This result may indicate that this dose approaches the minimum threshold necessary to achieve the full sterilization of *Ae. albopictus* females and that slight modifications of the experimental set up are sufficient to induce the preservation of a small percentage of fertile eggs. An example of this has recently been reported by Zheng et al. [23] who stated that, during remarkable open field trials based on a SIT/IIT combined strategy, a dose of 45 Gy might be necessary to achieve the sterilization of the females escaping the sexing procedures instead of the 28 Gy dose which had been demonstrated as sufficient at the laboratory scale [22]. Despite this, a few fertile eggs of the released and previously *Wolbachia*-transinfected HC *Ae. albopictus* were found in the ovitraps used to monitor the results of the experimentation. This evidence highlights the possible significant differences between the laboratory conditions and large-scale operational conditions, and the need for studies specific to the latter being performed prior to conducting open field trials [49]. This is particularly necessary in relation to releases involving mosquito strains with altered *Wolbachia* infection types that have the ability to spread in the wild population thanks to a Uni-CI pattern [15]. In these cases, a few partially fertile females could initiate a local population replacement with unpredictable safety consequences. Specific replacement programs are based on the release of mosquito populations with diminished vector competence aimed at reducing the mean vectorial capacity of the wild-type populations [21, 50–52]. However, population replacement should be a planned goal and should not be a consequence of area-wide programs addressing only population suppression. The strategy to pursue a *Wolbachia*-based population replacement program should be based on the results of thorough studies performed to ascertain the stability of the positive effects associated with a specific *Wolbachia* infection type in the long term and under various environmental conditions [3, 53–56]. In this context, for safety purposes, adding a perfect sexing protocol to Uni-CI based IIT programs [20] should be preferable to combining IIT with SIT [22, 23, 49]. This second approach would avoid the release of fertile and potentially invasive females, but

the required radiation doses may be adequately high as to seriously compromise the male mating competitiveness [23] and may induce an enhanced biting behavior in the co-released females evidenced in this study.

The decrease in density of *Wolbachia* determined by the radiation treatment evidenced in this study adds further concerns regarding the safe application of SIT in insect species infected by this bacterium when perfect sexing methods are not performed.

The *Wolbachia*-mediated reduction of the vector competence in mosquitoes strictly depends on the bacterial titer and the specific strain of the bacterium [30, 57]. The density of *Wolbachia* in naturally infected *Ae. albopictus* is an individual feature that may vary largely within a population [38] and that may be affected by the environmental conditions (temperature and food availability) under which the larvae develop [54-56]. Although distribution in somatic tissues has been observed in various cases, *Wolbachia* is mainly present within the germ line [58], and previous authors have already evidenced that the ovarian tissues of irradiated females are severely affected by the treatment [12, 22]. Bacteria are generally less sensitive to radiation compared to eukaryotes [59]; nevertheless, the density of *Wolbachia* is markedly affected by radiation doses applied to other insect species to achieve sterilization [28]. The results presented in this study highlighted an overall decrease in the density of *Wolbachia* in irradiated females, and this decrease was negatively correlated to the age of the pupae at the time of the radiation treatment and positively correlated to female aging. This downward trend was common in all the studied *Wolbachia* strains when compared to the untreated controls.

Early irradiation is generally associated with a stronger efficiency of the sterilization treatment [37]; however, negative effects on fitness have been found to increase with younger treated pupae. For this reason, SIT programs generally choose an intermediate pupal age to achieve full sterilization and to sufficiently preserve adult male fitness [12]. The damage experienced by the ovaries is consistent with the complete sterilization occurring when sufficiently high doses are applied. Our results highlighted that the destruction of these tissues, which are relatively sensitive to radiation due to intense mitotic activity [60], seems to also affect the survival and/or the reproduction of the hosted *Wolbachia*. The endosymbiont may be involved, together with the host cells, in the mechanisms of apoptosis usually characterizing the oocytes subjected to irradiation [61]. However, further studies should be conducted to investigate this phenomenon and to determine why the reduction in the density of the bacterium becomes evident by qPCR only over a week after the treatment. This occurrence may be partly explained by a gradual clearance of the bacterial DNA corresponding to dead individuals or present in extracellular form [62] in the damaged ovarian tissues of irradiated females. Selection of an opportune method to normalize the qPCR data

obtained after analysis of the ovaries has remained a fundamental experimental method to identify this event because irradiation has been demonstrated to compromise the suitability of various common housekeeping genes when tissues are damaged [63]. The comparison of the actin gene copy number in the ovaries of irradiated and control individuals confirms the hypothesis (S1 Fig.), and suggests the conduction of specific studies to identify the best candidate housekeeping gene or compliance with the method of normalization described in this study when performing similar experiments.

FISH analysis results supported the qPCR data related to ovaries, as demonstrated by the markedly reduced *Wolbachia*-specific fluorescence intensity associated with these tissues in irradiated females aged 13 ± 1 days. This evidence reinforces the idea that the population of this endosymbiotic bacterium is severely affected by a 45-Gy radiation treatment, at least in this organ, and that qPCR was only partially capable of highlighting this phenomenon.

Overall, the above-mentioned data are not sufficient to determine whether a radiation treatment can be associated with a loss of specific *Wolbachia*-controlled biological activities such as pathogen interference. *Wolbachia* endosymbionts mainly exert their limiting action on arbovirus dissemination in the bodies of *Aedes* species by acting in the midgut [64] and, similar to ovarian cells, midgut cells are characterized by intense mitotic activity and their structure and physiology have been previously shown to be affected by radiation [60, 65]. Therefore, we suggest that *Wolbachia* and the related host-symbiont mechanisms of regulation may be affected by radiation in these tissues as well. The apparent radiation-induced increase in the *Wolbachia* endosymbionts present in the extraovarian tissues (which include the midgut and salivary glands) of females aged 13 ± 1 days, even if not statistically significant, certainly deserves attention and further exploration. However, we have not provided evidence on the tissues specifically involved in these phenomena. Furthermore, as reported above, detection of *Wolbachia* DNA does not necessarily indicate that the bacterium is alive or capable of fully exerting its effects on host physiology, because nucleic acid-based analytical methods provide only limited information on the activities and physiological state of microorganisms in samples. These aspects can be detected retrospectively, but only after sufficient time has elapsed for the degradation and removal of DNA associated with inactivated cells [62, 66].

A qPCR analysis specifically targeting the *Wolbachia* density in the gut of irradiated females may add useful information to investigate the population dynamics of these bacteria in these tissues. However, for safety reasons, specific vector-competence studies would be decisive to ascertain whether a sterilizing radiation treatment may implicate a significantly increased risk of virus acquisition or transmission per single bite, and if the results can be coupled with the enhanced

biting activity data evidenced by the present study. For such a study to be viable, oral infection trials should be conducted on mass-reared mosquito females prior to performing irradiation at the doses necessary for large-scale operational programs, and all *Wolbachia*-infected vector species should be included [49].

In the case of programs based on the combination of SIT and IIT, investigation of the effects of irradiation on the induced level of CI in treated males should also be performed, since based on the results of the ovaries, a decrease in the titer of *Wolbachia* in the testes following the irradiation is a reasonably possible scenario.

Conclusion

The presented results reinforce the idea that performing radiation as a strategy to achieve male sterilization would enable the utilization of perfect sexing methods necessary to avoid the release of any vector female with markedly increased biting activity and sufficiently preserved fitness. Even a low percentage of contaminating mosquito females may result in the release of thousands of boosted vectors under large scale settings. In our opinion, this risk coupled with the complexity of the SIT framework and the markedly reduced male mating competitiveness induced by the radiation doses required in large-scale settings [23] may discourage the application of radiation methods when other efficient and safe methods to achieve male sterility are available. Particularly, with the advent of advanced, automated and efficient systems of sex separation capable of preventing the escape of fertile females during release of sterile males [20, 67, 68], the most relevant concern related to the *Wolbachia*-based suppression strategies is eliminated. In fact, the establishment in nature of mosquito lines with altered *Wolbachia* infection, which may compromise the long-term efficiency of IIT strategy, will be prevented by combining superior sexing procedures with opportune release programmes [69] in the case of both Uni-CI and Bi-CI patterns. In the latter case, the above-mentioned measures will be particularly effective because Bi-CI lowers the chance for the establishment of released mosquitoes in the field [15, 18, 49].

This evidence represents a fundamental step in the development of efficient, sustainable and safe strategies for vector control.

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Figure Captions

Figure 1. Kaplan-Meier survival curves comparing, respectively, irradiated and untreated S_{ANG} (A) and ARwP (B) *Ae. albopictus* over two weeks since the emergence. Differences between treatments were not statistically significant (Log rank test analysis: $P < 0.05$).

Figure 2. Altered biting behavior in irradiated *Ae. albopictus* and *Ae. aegypti*. The experiment was carried out in laboratory cages by offering daily a blood meal over two sets of 5 subsequent days interrupted by 2 days of rest and starting with 5 days old females. **A:** Daily mean percentage of biting S_{ANG} and ARwP *Ae. albopictus*; **B:** Mean weekly number of bites per S_{ANG} and ARwP *Ae. albopictus* female; **C:** Daily mean percentage of biting *Ae. aegypti*; **D:** Mean weekly number of

bites per *Ae. aegypti* female; A and C: within each mosquito population, different letters indicate statistically significant differences between treatments by Repeated measures ANOVA followed by Tukey's HSD test ($P < 0.05$); B and D: within each mosquito population, different letters indicate statistically significant differences between treatments (One-Way ANOVA followed by Tukey's test: $P < 0.05$).

Figure 3. Altered host seeking and biting behavior in irradiated S_{ANG} and ARwP *Ae.*

***albopictus* under large enclosures setting.** The experiment was carried out in Large Experimental Units ($8.5 \times 5 \times 5$ m) under open field climatic conditions and involved females irradiated at 45-Gy compared to untreated counterparts. Average landing time and biting proportions were compared between treatments within a 15 minutes interval. **A:** Comparison between irradiated and untreated starved females (6 ± 1 days old); **B:** Comparison between irradiated and untreated females 48 hours after the engorgement (8 ± 1 days old). Two-way ANOVA demonstrated that the difference between treatments was statistically significant in the case of the engorged females ($P < 0.05$).

Figure 4. Decreased *Wolbachia* titer in irradiated S_{ANG} and ARwP *Ae. albopictus*. **A:** A 45-Gy dose was applied at three different pupal ages (26, 36 and 46 ± 2 hours); quantitative PCR analysis was then performed by using primers targeting the specific *Wolbachia* strains characterizing the two *Ae. albopictus* populations and analyzing 6 ± 1 days old females. **B:** A 45-Gy dose was applied to pupae aged 36 ± 4 hours; qPCR analysis was then carried out studying two female ages (6 and 13 ± 1 days). Data were normalized by using the actine gene as host reference. Within each infection type and female age, different letters indicate statistically significant differences between treatments (One-way ANOVA followed by Tukey's test : $P < 0.05$).

Figure 5. Decreased *Wolbachia* titer in the ovaries of irradiated S_{ANG} and ARwP *Ae.*

***albopictus*.** A 45-Gy dose was applied to 36 ± 4 hours old pupae. **A:** qPCR analysis was performed on the dissected ovaries of 6 and 13 ± 1 days old females by using primers targeting the specific *Wolbachia* strains characterizing the two *Ae. albopictus* populations. Data were normalized by using DNA. **B:** qPCR analysis was performed on the deovarized tissues of 6 and 13 ± 1 days old females by using primers targeting the specific *Wolbachia* strains characterizing the two *Ae. albopictus* populations. Data were normalized by using the actine gene as host reference. Within each infection type and female age, different letters indicate statistically significant differences between treatments (One-way ANOVA followed by Tukey's test : $P < 0.05$).

Figure 6. Fluorescence In Situ Hybridization of *Ae. albopictus* ovaries in irradiated females. A 45-Gy dose was applied to 36±4 hours old pupae of S_{ANG} and AR_{WP} *Ae. albopictus*. Ovaries from sample individuals aged 13±1 days were then subjected to FISH analysis and compared with untreated counterparts. The distribution of *Wolbachia* is evidenced in green while blue stain is DAPI. **A:** Ovaries of untreated S_{ANG} females; **B:** Ovaries of irradiated S_{ANG} females **C:** Ovaries of untreated AR_{WP} females; **D:** Ovaries of irradiated AR_{WP} females.

Supporting Information

S1 Fig. Actine gene copies in the ovaries of S_{ANG} *Ae. albopictus* females irradiated at 45 Gy and then analyzed by qPCR when aged 6±1 and 13±1 days in comparison with untreated counterparts.

S2 Fig. Host seeking and biting behavior of irradiated S_{ANG} and AR_{WP} *Ae. albopictus* under large enclosures compared to untreated controls. Biting proportions and average times to landing were compared between treatments within a 15 minutes interval. **A:** Comparison between untreated and irradiated starved females aged 13±1 days; **B:** Comparison between irradiated and untreated females engorged females 48 hours after the engorgement (i.e. 15±1 days old). Two-way ANOVA demonstrated that the difference between treatments was statistically significant in the case of the engorged females ($P < 0.05$).

S3 Fig. Structural damage induced by irradiation at 45 Gy in the ovaries of 13±1 days old *Ae. albopictus* females in bright field. **A:** Ovaries of untreated S_{ANG} females; **B:** Ovaries of irradiated S_{ANG} females **C:** Ovaries of untreated AR_{WP} females; **D:** Ovaries of irradiated AR_{WP} females.

S4 Fig. FISH analysis of the ovaries of 13±1 days old *Ae. albopictus* belonging to a line (AR) cured from *Wolbachia* infection. **A** and **D:** DAPI stained; **B** and **E:** FITC stained; **C** and **F:** bright field. No specific green-fluorescent signal was detected in the aposymbiotic line.

S5 Fig. Additional images related to the FISH analysis of the ovaries of 13±1 days old S_{ANG} *Ae. albopictus* irradiated at 45 Gy. The distribution of *Wolbachia* is evidenced in green while blue stain is DAPI. **A** and **D:** DAPI stained; **B** and **E:** FITC stained; **C** and **F:** bright field. The green-fluorescent signal related to *Wolbachia* is weak and not homogeneously distributed.

S6 Fig. Additional images related to the FISH analysis of the ovaries of 13±1 days old ARwP *Ae. albopictus* irradiated at 45 Gy. The distribution of *Wolbachia* is evidenced in green while blue stain is DAPI. **A** and **D**: DAPI stained; **B** and **E**: FITC stained; **C** and **F**: bright field. The green-fluorescent signal related to *Wolbachia* is weak and not homogeneously distributed.

S7 Fig. Additional images related to the FISH analysis of the ovaries of 13±1 days old untreated ARwP *Ae. albopictus*. Blue stain is DAPI. **A** and **D**: DAPI stained; **B** and **E**: FITC stained; **C** and **F**: bright field. The green-fluorescent signal related to *Wolbachia* is strong and regularly distributed.

S8 Fig. Additional images related to the FISH analysis of the ovaries of 13±1 days old untreated S_{ANG} *Ae. albopictus*. Blue stain is DAPI. **A** and **D**: DAPI stained; **B** and **E**: FITC stained; **C** and **F**: bright field. The green-fluorescent signal related to *Wolbachia* is strong and regularly distributed.

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