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 extraovaric 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 verifes the inefficacy of the current control methods [4–6].

The negtive 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. Futhermore, 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

168 [30]. Herein, we aimed to further the knowledge by determining whether irradiation interfered with
169 host-biting and host-seeking behaviors in *Ae. albopictus* and *Ae. aegypti* to comprehensively
170 evaluate the potential of application of SIT or SIT/IIT strategies in the absence of a perfect
171 procedure for sexing. Furthermore, the effects of the radiation on the titer of *Wolbachia* in *Ae.*
172 *albopictus* were evaluated in whole mosquito bodies by qPCR, applying the treatment at different
173 pupal ages and analyzing the effects at two adult ages. Additionally, ovaries and extraovaric tissues
174 were similarly studied. Finally, FISH analysis was also conducted on irradiated ovaries to visualize
175 the effects of the treatment on the bacterial population and to acquire further useful information for
176 the interpretation of the results. Two *Ae. albopictus* lines have been used in the experiments to
177 determine whether different *Wolbachia* strains might affect the results. The resultant data will be
178 useful for comparing IIT, SIT and SIT-IIT combined strategies in terms of safety and sustainability,
179 and to more consciously evaluate their applicability on a large scale.
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183 ## Materials and methods

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

186

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

202 Bioethical Committee according to the EU directive 2010/63/EU. The mice belonged to a colony
203 housed at CR ENEA Casaccia and maintained for experimentation based on the authorization N.
204 80/2017-PR released on February 2, 2017 by the Italian Ministry of Health. Blood feeding by
205 female mosquitoes on human volunteers during the experiments was also approved by the ENEA
206 Bioethical Committee, and only a few of the Authors (RM, EL, GL, and MC) were involved.
207

208

209 **Radiation methods**

210 Cohorts of *Ae. albopictus* and *Ae. aegypti* females belonging to the strains described above were
211 irradiated with X-rays to enable their comparison with sham-exposed individuals (control).
212 X-ray radiation was performed using the Gilardoni CHF 320G X-ray generator (Gilardoni S.p.A.;
213 Mandello del Lario, Lecco, Italy) operated at 250 kVp, and 15 mA, with filters of 2.0 mm of Al and
214 0.5 mm of Cu, furnished by the Physical Technologies for Security and Health Division of ENEA.
215 Depending on the experiment and according to doses already tested for the radiation-based
216 sterilization of the two species [12, 22, 23], 28, 35, and 45 Gy (dose rate: 0.868 ± 0.004 Gy/min,
217 mean ± SD) were applied to *Ae. albopictus* pupae, while a single dose of 50 Gy was used to treat
218 *Ae. aegypti*, since this dosage is known to fully inhibit egg production in the species [12]. Groups of
219 100 female pupae were transferred into a Petri dish (d= 4 cm) at 36±4 hours of age (unless specified
220 differently) and then transported to the irradiation facility. Immediately before commencement of
221 irradiation, most of the residual water was removed using a glass pipette; irradiated pupae were then
222 transferred in a larger water container to facilitate complete development and allow for adult
223 emergence inside the experimental cages. Sham-exposed pupae, that is pupae treated as the exposed
224 pupae excepted for X-ray exposure, were used as controls.

225

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

227 Females irradiated at 28, 35, and 45 Gy, and untreated counterparts belonging to S_{ANG} or AR*w*P *Ae.*
228 *albopictus* were allowed to emerge inside 30 × 30 × 30 cm plastic cages which were checked for the
229 presence of males that escaped the sexing procedure [19]. The number of females in each cage was
230 adjusted to constitute a cohort of 30 virgin individuals to which 30 untreated males, characterized
231 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*w*P 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

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

284

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

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

297 effects on the bacterium in the germ line and somatic tissues. Untreated females were used as
298 control.
299 DNA was extracted from single individuals using the ZR Tissue & Insect DNA Kit MicroPrep
300 (Zymo Research, Irvine, CA, USA), according to manufacturer's instructions. When necessary,
301 females were preliminarily chilled on ice and dissected using phosphate-buffered saline (PBS) to
302 allow for the isolation of the ovaries from the other tissues, excluding the individuals with ovaries
303 that were not intact.
304 Real-time PCR was performed using the Roche LightCycler® 96 Instrument (Roche Molecular
305 Systems, Inc., Rotkreuz, Switzerland). Each reaction was performed in triplicate with a 20-µL final
306 volume containing 10 µL of the Luna® Universal qPCR Master Mix (New England BioLabs,
307 Ipswich, MA, USA), 0.03 µL of 150 nM each primer, and 2 µL of purified DNA using the
308 following amplification program: initial activation at 95 °C for 120 s, followed by 40 cycles at 95
309 °C for 15 s and 60 °C for 40 s. The presence of specific amplification products was verified using
310 dissociation curves [38].
311 Strain-specific primers were used to amplify the *wsp* loci [39], namely, the *w*AlbA-*wsp* and *w*AlbB-
312 *wsp* loci in the case of S_{ANG} females, and the *w*Pip-*wsp* loci in the case of AR*w*P specimens. *Wsp*
313 plasmid standards were used to generate a standard curve for absolute quantification [38]. The *Ae.*
314 *albopictus* actin gene was used as a nuclear reference of whole body extracts and extracts obtained
315 after the removal of the ovaries and was amplified with the primer pair actAlbqPCR [40]. Owing to
316 the marked decrease in actin gene copies observed preliminarily in irradiated ovaries (S1 Fig.), the
317 normalization of qPCR data related to these organs was performed using total DNA content as
318 reference [41]. For this purpose, the Thermo Scientific NanoDrop 2000 spectrophotometer (Thermo
319 Fisher, Waltham, MA, USA) was used.
320

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

322 Based on the results of qPCR, fluorescent in situ-hybridization (FISH) analysis was conducted on
323 the ovaries of 13±1-day-old irradiated and untreated females according to the protocol described by
324 previous authors [42]. Two hundred nanograms of the *Wolbachia* specific 16S rRNA probe (W2:
325 5′-CTTCTGTGAGTACCGTCATTATC-3′) was added in the hybridization buffer [43]. Tissues
326 were placed on a slide containing a drop of the VECTASHIELD Antifade Mounting Medium with
327 DAPI (Vector Laboratories, Burlingame, CA, USA) and visualized using the Nikon Eclipse E800
328 confocal microscope and NIS-Elements 4.0 software (Nikon, Tokyo, Japan). An aposymbiotic
329 population of *Ae. albopictus* [31] was used as the negative control.

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332 **Data analysis**

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334 Results were expressed as mean ± SE, and the arcsin square root transformation was applied to
335 analyze proportional data. The Levene's test and the Shapiro-Wilk test were performed to assess
336 equality of variances and normality, respectively. Statistical analysis was performed using the
337 software PASW statistics (PASW Statistics for Windows, Version 18.0. SPSS Inc., Chicago, IL,
338 USA) with the level of significance set at $P < 0.05$.
339 The survival curves of the four different treatments for each *Wolbachia* infection type, were
340 compared using the Kaplan-Meier method and the log-rank (Mantel-Cox) test. The Kruskal-Wallis
341 H-test followed by the Conover-Iman test was used to compare fecundity and egg hatch data
342 obtained between treatments within each infection type.
343 Repeated measures ANOVA was used to analyze the bite data obtained between treatments over
344 each week. Cages were considered the experimental units and data have been expressed as the mean
345 percentage of engorged females per day, adjusted for cage-specific mortality. If Mauchly's test
346 indicated a violation of the sphericity assumption, the degrees of freedom were corrected by using
347 Huynh-Feldt estimates. Multiple comparisons between treatments were assessed using Tukey's
348 HSD post hoc test. Additionally, one-way ANOVA was performed to compare the mean number of
349 bites per female per treatment during each week of study.
350 Data regarding the host-seeking behavior under large enclosures were analyzed by assigning a value
351 to the mosquitoes a value based on the median time of each catching interval to compute the
352 average landing time. The proportion of engorged mosquitoes was measured considering the ones
353 retrieved within the defined 15-min interval. Two-way ANOVA was used to analyze the differences
354 between groups in terms of the proportions of females that had bitten the host and to compare
355 average landing times. The Shapiro-Wilk test was conducted to ascertain that the proportions and
356 average landing times were normally distributed.
357 One-way ANOVA was used to compare, within each *Wolbachia* strain, the qPCR data obtained
358 from *Ae. albopictus* females treated at the three tested pupal ages or untreated. The effect of female
359 aging on the overall titer of *Wolbachia* was also analyzed by performing one-way ANOVA within
360 data of each strain of the bacterium. Data regarding ovaries and extraovaric tissues of the treated or
361 untreated counterparts were similarly analyzed at the two tested female ages. In the case of rejection
362 of the assumptions of equality of variance and/or normality, the Kruskal-Wallis rank-sum test was
363 performed.

364

365

366 # Results

367

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

369

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

378

379 **Table 1.** Fecundity and fertility of AR*w*P and S_{ANG} *Ae. albopictus* females irradiated at 28, 35 and 45 Gy
380 compared to untreated counterparts over two gonotrophic cycles. Fecundity and egg hatching data related to
381 *Ae. aegypti* irradiated at 50 Gy are also reported.
382

Mosquito line	Blood meal	Treatment	Mean (±SE) eggs per female	Mean (±SE) egg hatch (%) from the laid eggs
ARwP	1st	untreated	31.54 ± 0.77 a	85.87 ± 2.37 a
		28 Gy	5.67 ± 0.36 b	13.72 ± 1.77 b
		35 Gy	0.11 ± 0.04 c	0.00 ± 0.00 c
		45 Gy	0.00 ± 0.00 d	0.00 ± 0.00 c
	2nd	untreated	30.89 ± 0.79 a	85.61 ± 2.28 a
		28 Gy	5.04 ± 0.46 b	15.03 ± 1.91 b
		35 Gy	0.15 ± 0.04 c	0.00 ± 0.00 c
		45 Gy	0.00 ± 0.00 d	0.00 ± 0.00 c
S_{ANG}	1st	untreated	32.24 ± 0.82 a	87.44 ± 2.38 a
		28 Gy	5.45 ± 0.44 b	14.75 ± 2.28 b
		35 Gy	0.13 ± 0.04 c	0.00 ± 0.00 c
		45 Gy	0.00 ± 0.00 d	0.00 ± 0.00 c
	2nd	untreated	31.44 ± 0.79 a	86.77 ± 2.40 a
		28 Gy	5.27 ± 0.38 b	15.25 ± 1.60 b
		35 Gy	0.13 ± 0.03 c	2.86 ± 2.86 c
		45 Gy	0.00 ± 0.00 d	0.00 ± 0.00 d
Ae. aegypti	1st	50 Gy	0.00 ± 0.00	0.00 ± 0.00
	2nd	50 Gy	0.00 ± 0.00	0.00 ± 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
383 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*w*P-1st wk: Sphericity assumed, $F_{(12,}$
390 $_{64)} = 5.90$, $P < 0.05$; AR*w*P-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*w*P
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 ±
415 0.04 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

418

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

420

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

423 The host-seeking behavior exhibited by irradiated females under large enclosure conditions
424 reinforced the results obtained in small cages. The applied X-ray dose was not sufficient to reduce
425 the ability of *Ae. albopictus* to reach the host in the LEU compared to untreated females (Fig.3A,
426 B). The average landing times were not significantly affected by the infection type (two-way
427 ANOVA: $F_{1,20} = 0.66$, $P = 0.43$), nor were they affected by the treatment (two-way ANOVA: $F_{1,20} =$
428 0.93, $P = 0.35$) in starved females. On average, landing times (±SE) were, respectively, 346.31 ±
429 15.98 and 354.30 ± 10.74 in irradiated and untreated S_{ANG} females and 331.11 ± 12.74 and 348.29 ±
430 12.30 in irradiated and control AR*w*P individuals.

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

443

444

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

446

447 The age at which *Ae. albopictus* pupae had been irradiated at 45 Gy was found to affect significantly
448 the density of these bacteria (*w*AlbA: $F_{(3, 36)}$=2.96, P < 0.05; *w*AlbB: $F_{(3, 36)}$=4.05, P < 0.05; *w*Pip: $F_{(3,}$
449 $_{36)}$=6.76, P < 0.05) regardless of the *Wolbachia* infection type. An early irradiation (26±2-hour-old
450 pupae) induced a significant decrease in the whole body *Wolbachia* titer approximating a third in
451 *w*AlbA, and a half in *w*AlbB and *w*Pip strains (Fig. 4A) compared to the levels observed in the
452 controls. As evidenced by the pairwise comparisons, the *Wolbachia* titer between treated and untreated
453 individuals did not significantly differ when irradiation was performed on older pupae (36 and 46±2
454 hours old).
455 Adult female aging was also found to affect the titer of all the tested *Wolbachia* strains when a 45-Gy
456 radiation treatment was performed (Fig. 4B). The difference between treatments was not statistically
457 significant when the analysis had been performed on 6±1-day-old females (one-way ANOVA; *w*AlbA:
458 $F_{(1, 18)}$ = 1.54, P = 0.23; *w*AlbB: $F_{(1, 18)}$ = 0.82, P = 0.89; *w*Pip: $F_{(1, 18)}$ = 3.28, P = 0.09), but the decrease
459 increased significantly in females aged 13±1 days (one-way ANOVA; *w*AlbA: $F_{(1, 18)}$ = 62.11, P < 0.05;
460 *w*AlbB: $F_{(1, 18)}$=38.93, P < 0.05; *w*Pip: $F_{(1, 18)}$=4.60, P < 0.05).
461 In agreement with the data obtained from the analysis of whole bodies, results of qPCR analysis also
462 evidenced a correlation between aging and a reduced titer of the bacteria in the ovaries of irradiated
463 compared to untreated *Ae. albopictus* females (Fig. 5A). This phenomenon was prominent when
464 conducting analysis of females at 13±1 days after the irradiation (one-way ANOVA; *w*AlbA: $F_{(1, 18)}$ =
465 62.11, P < 0.05; *w*AlbB: $F_{(1, 18)}$ = 5.91, P < 0.05; *w*Pip: $F_{(1, 18)}$ = 4.60, P < 0.05), whereas the *Wolbachia*
466 density did not differ between treatments in 6±1-day-old females with regard to *w*AlbA and *w*AlbB
467 *Wolbachia* strains (one-way ANOVA; *w*AlbA: $F_{(1, 18)}$ = 0.39, P = 0.54; *w*AlbB: $F_{(1, 18)}$ = 0.50, P = 0.49).
468 A significant difference was found between the *w*Pip *Wolbachia* titer in treated and untreated 6±1-day-
469 old AR*w*P females ($F_{(1, 18)}$ = 5.25, P < 0.05). As also shown in the Figure 5A, the titer of *w*AlbA and
470 *w*AlbB *Wolbachia* increased with aging in the ovaries of control females and confirmed previous
471 studies [38], while irradiation led to an inversion of this trend. Regarding *w*Pip, the controls evidenced
472 an age-dependent decrease in titer and irradiation further intensified this trend in the density of the
473 bacteria.
474 When testing the carcasses of the females without ovaries, the qPCR amplification results of
475 *Wolbachia* (somatic fraction) did not reveal differences between treatments related to the titers of

476 *w*AlbA, *w*AlbB and *w*Pip *Wolbachia* when irradiated females had been tested at 6±1 days (Fig. 5B)
477 (Kruskal Wallis test; *w*AlbA: $\chi2 = 1.85$, df = 1, P = 0.17; *w*AlbB: $\chi2 = 0.32$, df = 1; P = 0.57; *w*Pip: $\chi2$
478 = 1.37, df = 1, P = 0.24). In contrast with the results obtained in the ovaries, the titer of *Wolbachia* in
479 the extraovaric tissues eemed to increase with aging in irradiated females compared to the controls but
480 differences between treatments were not statistically significant (Kruskal Wallis test; *w*AlbA: $\chi2$ =
481 2.17, df = 1, P = 0.14; *w*AlbB: $\chi2 = 2.52$, df = 1, P = 0.11; *w*Pip: $\chi2 = 0.24$, df = 1, P =0.62).
482

483 **FISH analysis results**

484

485 Quantitative PCR results related to the investigation of the ovaries were consistent with the images
486 obtained by FISH analysis that showed occurrence of evident histological damage due to radiation with
487 consequent reduction in the mean size of the organs (S3 Fig.), coupled with a markedly reduced
488 *Wolbachia*-specific fluorescence intensity (Fig. 6, S4 Fig., S5 Fig., S6 Fig.). The analysis of the
489 *Wolbachia*-infected individuals showed that fluorescent signals differed markedly between treatments
490 (Fig. 6). In most of the ovaries dissected from S_{ANG} and AR*w*P females irradiated at 45 Gy, a weak
491 fluorescent signal was observed (Fig. 6B, 6D, S5 Fig., S6 Fig.) compared to untreated controls (Fig.
492 6A, 6C, S7 Fig., S8 Fig). In certain cases, the fluorescence was only localized in the ovarioles that
493 possibly had not been degenerated by the irradiation (Fig. 6D, red circle). In contrast, the organs
494 maintained their authentic ovariole structure in untreated samples and the signal was abundant,
495 specific, and confined within each oocyte (Fig. 6, S7 Fig., S8 Fig.).
496
497

498 ## Discussion

499 Several area-wide methods for mosquito control are under investigation based on the release of
500 conspecific male individuals capable of reducing the reproductive potential of the wild-type
501 populations through various mechanisms [7]. All these methods target specificity, efficacy, safety,
502 and sustainability, in contrast to the traditional chemical-based methods and have demonstrated
503 specific positive and negative aspects. Common to these strategies is the need to curb the release of
504 a significant proportion of females and to prevent issues in the vector control strategies, failure to
505 do which may potentially increase the load of vectors and the number of modified individuals
506 capable of reproduction in the field. This work highlights how application of superior methods for
507 sexing before release may be fundamental when males are sterilized through the application of
508 radiation treatments.
509 Multiple blood-feeding is known to occur in *Ae. albopictus* as not all of the ovarian follicles exhibit
510 commencement of the gonotrophic cycle with a single blood meal [44]. Studies conducted on
511 engorged *Ae. aegypti* females demonstrated that they were generally inhibited from host-seeking by
512 the occurrence of distension-induced and oocyte-induced mechanisms of regulation [25, 45];
513 however, this inhibition does not regard all individuals [44]. Results presented here demonstrate
514 that the radiation doses characterizing SIT programs attenuate significantly this inhibition in both
515 *Ae. albopictus* and *Ae. aegypti*, and that only the distension-induced mechanism of control may
516 remain at least partially active. In fact, most females irradiated at 35 and 45 Gy gorged several
517 times a week, even if the highest number of bites was generally observed on the first day of blood
518 meal availability. Additionally, the dose of 28 Gy was found to be sufficient to induce a markedly
519 enhanced biting behavior compared to the untreated controls. The tissue alterations experienced by
520 the ovaries due to irradiation might explain this phenomenon as the host-seeking behavior is
521 interrupted through the release in the hemocoel of hormones produced by the ovaries with
522 oogenesis [46, 47]. In virus-endemic areas, multiple blood feeding indicates the existence of
523 multiple occasions for a female mosquito to be infected by a suitable arbovirus and this possibility
524 may remarkably increase the chances of virus transmission to other hosts. Furthermore, the
525 comparison of the survival curves of irradiated and untreated females revealed that the tested
526 irradiation doses were incapable of inducing a marked decrease in life expectancy within the first 20
527 days in *Ae. albopictus*, which is an age that only a minority of adults are expected to exceed in
528 nature [48]. This evidence implies that irradiated *Ae. albopictus* females escaping the sexing
529 procedure may have a stronger vectorial capacity compared to wild-type females, if fitness is
530 sufficiently preserved. Previously, flight ability in radiation-sterilized males was not reported to be

531 affected when compared to untreated males [12]. The experiments testing the host-seeking ability
532 under large enclosure conditions reported herein confirm that this biological feature is not affected
533 by radiation doses that are capable of fully compromising fecundity in females. Indeed, starved
534 females irradiated at 45 Gy did not exhibit a significantly different ability to reach and bite the host
535 compared to the controls, but when engorged, their behavior changed and they continued to seek
536 hosts, unlike the untreated females, leading to a remarkable increase in the mean number of bites
537 per individual.
538 Overall, dose response curves related to fecundity and fertility showed agreement with the results of
539 other studies [12] and verified the success of the radiation treatment. However, a dose of 28 Gy was
540 incapable of inducing full sterilization of the produced eggs as was reported by previous authors
541 [22]. This result may indicate that this dose approaches the minimum threshold necessary to achieve
542 the full sterilization of *Ae. albopictus* females and that slight modifications of the experimental set
543 up are sufficient to induce the preservation of a small percentage of fertile eggs. An example of this
544 has recently been reported by Zheng et al. [23] who stated that, during remarkable open field trials
545 based on a SIT/IIT combined strategy, a dose of 45 Gy might be necessary to achieve the
546 sterilization of the females escaping the sexing procedures instead of the 28 Gy dose which had
547 been demonstrated as sufficient at the laboratory scale [22]. Despite this, a few fertile eggs of the
548 released and previously *Wolbachia*-transinfected HC *Ae. albopictus* were found in the ovitraps used
549 to monitor the results of the experimentation. This evidence highlights the possible significant
550 differences between the laboratory conditions and large-scale operational conditions, and the need
551 for studies specific to the latter being performed prior to conducting open field trials [49]. This is
552 particularly necessary in relation to releases involving mosquito strains with altered *Wolbachia*
553 infection types that have the ability to spread in the wild population thanks to a Uni-CI pattern [15].
554 In these cases, a few partially fertile females could initiate a local population replacement with
555 unpredictable safety consequences. Specific replacement programs are based on the release of
556 mosquito populations with diminished vector competence aimed at reducing the mean vectorial
557 capacity of the wild-type populations [21, 50–52]. However, population replacement should be a
558 planned goal and should not be a consequence of area-wide programs addressing only population
559 suppression. The strategy to pursue a *Wolbachia*-based population replacement program should be
560 based on the results of thorough studies performed to ascertain the stability of the positive effects
561 associated with a specific *Wolbachia* infection type in the long term and under various
562 environmental conditions [3, 53-56]. In this context, for safety purposes, adding a perfect sexing
563 protocol to Uni-CI based IIT programs [20] should be preferable to combining IIT with SIT [22, 23,
564 49]. This second approach would avoid the release of fertile and potentially invasive females, but

565 the required radiation doses may be adequately high as to seriously compromise the male mating
566 competitiveness [23] and may induce an enhanced biting behavior in the co-released females
567 evidenced in this study.
568 The decrease in density of *Wolbachia* determined by the radiation treatment evidenced in this study
569 adds further concerns regarding the safe application of SIT in insect species infected by this
570 bacterium when perfect sexing methods are not performed.
571 The *Wolbachia*-mediated reduction of the vector competence in mosquitoes strictly depends on the
572 bacterial titer and the specific strain of the bacterium [30, 57]. The density of *Wolbachia* in
573 naturally infected *Ae. albopictus* is an individual feature that may vary largely within a population
574 [38] and that may be affected by the environmental conditions (temperature and food availability)
575 under which the larvae develop [54-56]. Although distribution in somatic tissues has been observed
576 in various cases, *Wolbachia* is mainly present within the germ line [58], and previous authors have
577 already evidenced that the ovarian tissues of irradiated females are severely affected by the
578 treatment [12, 22]. Bacteria are generally less sensitive to radiation compared to eukaryotes [59];
579 nevertheless, the density of *Wolbachia* is markedly affected by radiation doses applied to other
580 insect species to achieve sterilization [28]. The results presented in this study highlighted an overall
581 decrease in the density of *Wolbachia* in irradiated females, and this decrease was negatively
582 correlated to the age of the pupae at the time of the radiation treatment and positively correlated to
583 female aging. This downward trend was common in all the studied *Wolbachia* strains when
584 compared to the untreated controls.
585 Early irradiation is generally associated with a stronger efficiency of the sterilization treatment [37];
586 however, negative effects on fitness have been found to increase with younger treated pupae. For
587 this reason, SIT programs generally choose an intermediate pupal age to achieve full sterilization
588 and to sufficiently preserve adult male fitness [12]. The damage experienced by the ovaries is
589 consistent with the complete sterilization occurring when sufficiently high doses are applied. Our
590 results highlighted that the destruction of these tissues, which are relatively sensitive to radiation
591 due to intense mitotic activity [60], seems to also affect the survival and/or the reproduction of the
592 hosted *Wolbachia.* The endosymbiont may be involved, together with the host cells, in the
593 mechanisms of apoptosis usually characterizing the oocytes subjected to irradiation [61]. However,
594 further studies should be conducted to investigate this phenomenon and to determine why the
595 reduction in the density of the bacterium becomes evident by qPCR only over a week after the
596 treatment. This occurrence may be partly explained by a gradual clearance of the bacterial DNA
597 corresponding to dead individuals or present in extracellular form [62] in the damaged ovarian
598 tissues of irradiated females. Selection of an opportune method to normalize the qPCR data

599 obtained after analysis of the ovaries has remained a fundamental experimental method to identify
600 this event because irradiation has been demonstrated to compromise the suitability of various
601 common housekeeping genes when tissues are damaged [63]. The comparison of the actin gene
602 copy number in the ovaries of irradiated and control individuals confirms the hypothesis (S1 Fig.),
603 and suggests the conduction of specific studies to identify the best candidate housekeeping gene or
604 compliance with the method of normalization described in this study when performing similar
605 experiments.
606 FISH analysis results supported the qPCR data related to ovaries, as demonstrated by the markedly
607 reduced *Wolbachia*-specific fluorescence intensity associated with these tissues in irradiated
608 females aged 13±1 days. This evidence reinforces the idea that the population of this endosymbiotic
609 bacterium is severely affected by a 45-Gy radiation treatment, at least in this organ, and that qPCR
610 was is only partially capable of highlighting this phenomenon.
611 Overall, the above-mentioned data are not sufficient to determine whether a radiation treatment can
612 be associated with a loss of specific *Wolbachia*-controlled biological activities such as pathogen
613 interference. *Wolbachia* endosymbionts mainly exert their limiting action on arbovirus
614 dissemination in the bodies of *Aedes* species by acting in the midgut [64] and, similar to ovarian
615 cells, midgut cells are characterized by intense mitotic activity and their structure and physiology
616 have been previously shown to be affected by radiation [60, 65]. Therefore, we suggest that
617 *Wolbachia* and the related host-symbiont mechanisms of regulation may be affected by radiation in
618 these tissues as well. The apparent radiation-induced increase in the *Wolbachia* endosymbionts
619 present in the extraovarian tissues (which include the midgut and salivary glands) of females aged
620 13±1 days, even if not statistically significant, certainly deserves attention and further exploration.
621 However, we have not provided evidence on the tissues specifically involved in these phenomena.
622 Furthermore, as reported above, detection of *Wolbachia* DNA does not necessarily indicate that the
623 bacterium is alive or capable of fully exerting its effects on host physiology, because nucleic acid-
624 based analytical methods provide only limited information on the activities and physiological state
625 of microorganisms in samples. These aspects can be detected retrospectively, but only after
626 sufficient time has elapsed for the degradation and removal of DNA associated with inactivated
627 cells [62, 66].
628 A qPCR analysis specifically targeting the *Wolbachia* density in the gut of irradiated females may
629 add useful information to investigate the population dynamics of these bacteria in these tissues.
630 However, for safety reasons, specific vector-competence studies would be decisive to ascertain
631 whether a sterilizing radiation treatment may implicate a significantly increased risk of virus
632 acquisition or transmission per single bite, and if the results can be coupled with the enhanced

633 biting activity data evidenced by the present study. For such a study to be viable, oral infection
634 trials should conducted on mass-reared mosquito females prior to performing irradiation at the
635 doses necessary for large-scale operational programs, and all *Wolbachia*-infected vector species
636 should be included [49].
637 In the case of programs based on the combination of SIT and IIT, investigation of the effects of
638 irradiation on the induced level of CI in treated males should also be performed, since based on the
639 results of the ovaries, a decrease in the titer of *Wolbachia* in the testes following the irradiation is a
640 reasonably possible scenario.
641

642 **Conclusion**

643 The presented results reinforce the idea that performing radiation as a strategy to achieve male
644 sterilization would enable the utilization of perfect sexing methods necessary to avoid the release of
645 any vector female with markedly increased biting activity and sufficiently preserved fitness. Even a
646 low percentage of contaminating mosquito females may result in the release of thousands of
647 boosted vectors under large scale settings. In our opinion, this risk coupled with the complexity of
648 the SIT framework and the markedly reduced male mating competitiveness induced by the radiation
649 doses required in large-scale settings [23] may discourage the application of radiation methods
650 when other efficient and safe methods to achieve male sterility are available. Particularly, with the
651 advent of advanced, automated and efficient systems of sex separation capable of preventing the
652 escape of fertile females during release of sterile males [20, 67, 68], the most relevant concern
653 related to the *Wolbachia*-based suppression strategies is eliminated. In fact, the establishment in
654 nature of mosquito lines with altered *Wolbachia* infection, which may compromise the long-term
655 efficiency of IIT strategy, will be prevented by combining superior sexing procedures with
656 opportune release programmes [69] in the case of both Uni-CI and Bi-CI patterns. In the latter case,
657 the above-mentioned measures will be particularly effective because Bi-CI lowers the chance for
658 the establishment of released mosquitoes in the field [15, 18, 49].
659 This evidence represents a fundamental step in the development of efficient, sustainable and safe
660 strategies for vector control.
661

662 **Acknowledgments**

663

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Figure Captions

Figure 1. Kaplan-Meier survival curves comparing, respectively, irradiated and untreated S_{ANG} (A) and AR*w*P (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 AR*w*P *Ae. albopictus*; **B:** Mean weekly number of bites per S_{ANG} and AR*w*P *Ae. albopictus* female; **C:** Daily mean percentage of biting *Ae. aegypti*; **D:** Mean weekly number of

701 bites per *Ae. aegypti* female; A and C: within each mosquito population, different letters indicate
702 statistically significant differences between treatments by Repeated measures ANOVA followed by
703 Tukey's HSD test ($P < 0.05$); B and D: within each mosquito population, different letters indicate
704 statistically significant differences between treatments (One-Way ANOVA followed by Tukey's
705 test: $P < 0.05$) .
706
707 **Figure 3. Altered host seeking and biting behavior in irradiated S_{ANG} and AR*w*P *Ae.*
708 ***albopictus* under large enclosures setting.** The experiment was carried out in Large Experimental
709 Units (8.5×5×5 m) under open field climatic conditions and involved females irradiated at 45-Gy
710 compared to untreated counterparts. Average landing time and biting proportions were compared
711 between treatments within a 15 minutes interval. **A:** Comparison between irradiated and untreated
712 starved females (6±1 days old); **B:** Comparison between irradiated and untreated females 48 hours
713 after the engorgement (8±1 days old). Two-way ANOVA demonstrated that the difference between
714 treatments was statistically significant in the case of the engorged females ($P < 0.05$) .
715
716 **Figure 4. Decreased *Wolbachia* titer in irradiated S_{ANG} and AR*w*P *Ae. albopictus.* A:** A 45-Gy
717 dose was applied at three different pupal ages (26, 36 and 46±2 hours); quantitative PCR analysis
718 was then performed by using primers targeting the specific *Wolbachia* strains characterizing the two
719 *Ae. albopictus* populations and analyzing 6±1 days old females. **B**: A 45-Gy dose was applied to
720 pupae aged 36±4 hours; qPCR analysis was then carried out studying two female ages (6 and 13±1
721 days). Data were normalized by using the actine gene as host reference. Within each infection type
722 and female age, different letters indicate statistically significant differences between treatments
723 (One-way ANOVA followed by Tukey's test : $P<0.05$).
724
725 **Figure 5. Decreased *Wolbachia* titer in the ovaries of irradiated S_{ANG} and AR*w*P *Ae.*
726 ***albopictus.*** A 45-Gy dose was applied to 36±4 hours old pupae. **A:** qPCR analysis was performed
727 on the dissected ovaries of 6 and 13±1 days old females by using primers targeting the specific
728 *Wolbachia* strains characterizing the two *Ae. albopictus* populations. Data were normalized by
729 using DNA. **B**: qPCR analysis was performed on the deovarized tissues of 6 and 13±1 days old
730 females by using primers targeting the specific *Wolbachia* strains characterizing the two *Ae.*
731 *albopictus* populations. Data were normalized by using the actine gene as host reference. Within
732 each infection type and female age, different letters indicate statistically significant differences
733 between treatments (One-way ANOVA followed by Tukey's test : $P<0.05$).
734

735 **Figure 6. Flourescence In Situ Hybridization of *Ae. albopictus* ovaries in irradiated females**. A
736 45-Gy dose was applied to 36±4 hours old pupae of S_{ANG} and AR*w*P *Ae. albopictus*. Ovaries from
737 sample individuals aged 13±1 days were then subjected to FISH analysis and compared with
738 untreated counterparts. The distribution of *Wolbachia* is evidenced in green while blue stain is
739 DAPI. **A**: Ovaries of untreated S_{ANG} females; **B**: Ovaries of irradiated S_{ANG} females **C**: Ovaries of
740 untreated AR*w*P females; **D**: Ovaries of irradiated AR*w*P females.
741
742
743 **Supporting Information**
744
745 **S1 Fig.** Actine gene copies in the ovaries of S_{ANG} *Ae. albopictus* females irradiated at 45 Gy and
746 then analyzed by qPCR when aged 6±1 and 13±1 days in comparison with untreated counterparts.
747
748 **S2 Fig.** Host seeking and biting behavior of irradiated S_{ANG} and AR*w*P *Ae. albopictus* under large
749 enclosures compared to untreated controls. Biting proportions and average times to landing were
750 compared between treatments within a 15 minutes interval. **A:** Comparison between untreated and
751 irradiated starved females aged 13±1 days; **B:** Comparison between irradiated and untreated
752 females engorged females 48 hours after the engorgement (i.e. 15±1 days old). Two-way ANOVA
753 demonstrated that the difference between treatments was statistically significant in the case of the
754 engorged females ($P < 0.05$).
755
756 **S3 Fig.** Structural damage induced by irradiation at 45 Gy in the ovaries of 13±1 days old *Ae.*
757 *albopictus* females in bright field**.** **A**: Ovaries of untreated S_{ANG} females; **B**: Ovaries of irradiated
758 S_{ANG} females **C**: Ovaries of untreated AR*w*P females; **D**: Ovaries of irradiated AR*w*P females.
759
760 **S4 Fig.** FISH analysis of the ovaries of 13±1 days old *Ae. albopictus* belonging to a line (AR) cured
761 from *Wolbachia* infection. **A** and **D**: DAPI stained; **B** and **E**: FITC stained; **C** and **F**: bright field.
762 No specific green-fluorescent signal was detected in the aposymbiotic line.
763
764 **S5 Fig.** Additional images related to the FISH analysis of the ovaries of 13±1 days old S_{ANG} *Ae.*
765 *albopictus* irradiated at 45 Gy. The distribution of *Wolbachia* is evidenced in green while blue stain
766 is DAPI. **A** and **D**: DAPI stained; **B** and **E**: FITC stained; **C** and **F**: bright field. The green-
767 fluorescent signal related to *Wolbachia* is week and not homogeneously distributed.
768

S6 Fig. Additional images related to the FISH analysis of the ovaries of 13±1 days old AR*w*P *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 week and not homogeneously distributed.

S7 Fig. Additional images related to the FISH analysis of the ovaries of 13±1 days old untreated AR*w*P *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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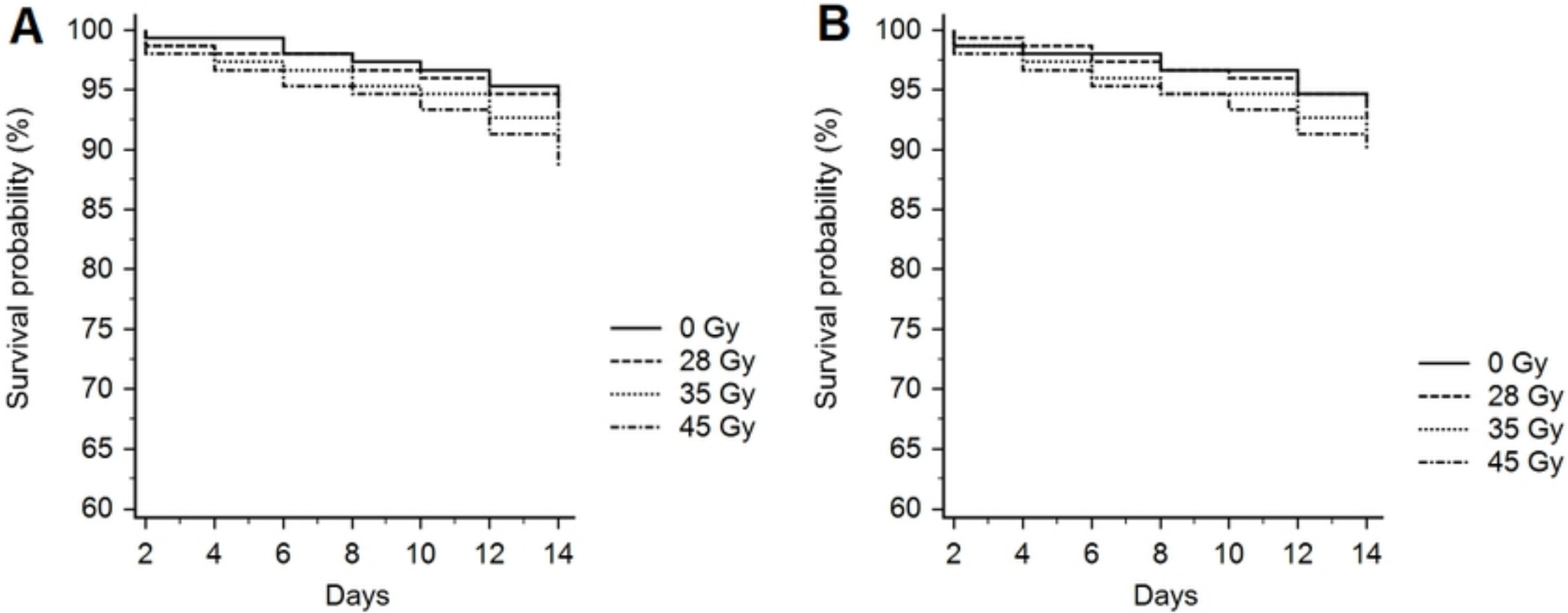
A
B
Survival probability (%)
100
95
90
85
80
75
70
65
60
2
4
6
8
10
12
14
Days
0 Gy
28 Gy
35 Gy
45 Gy

Figure 1

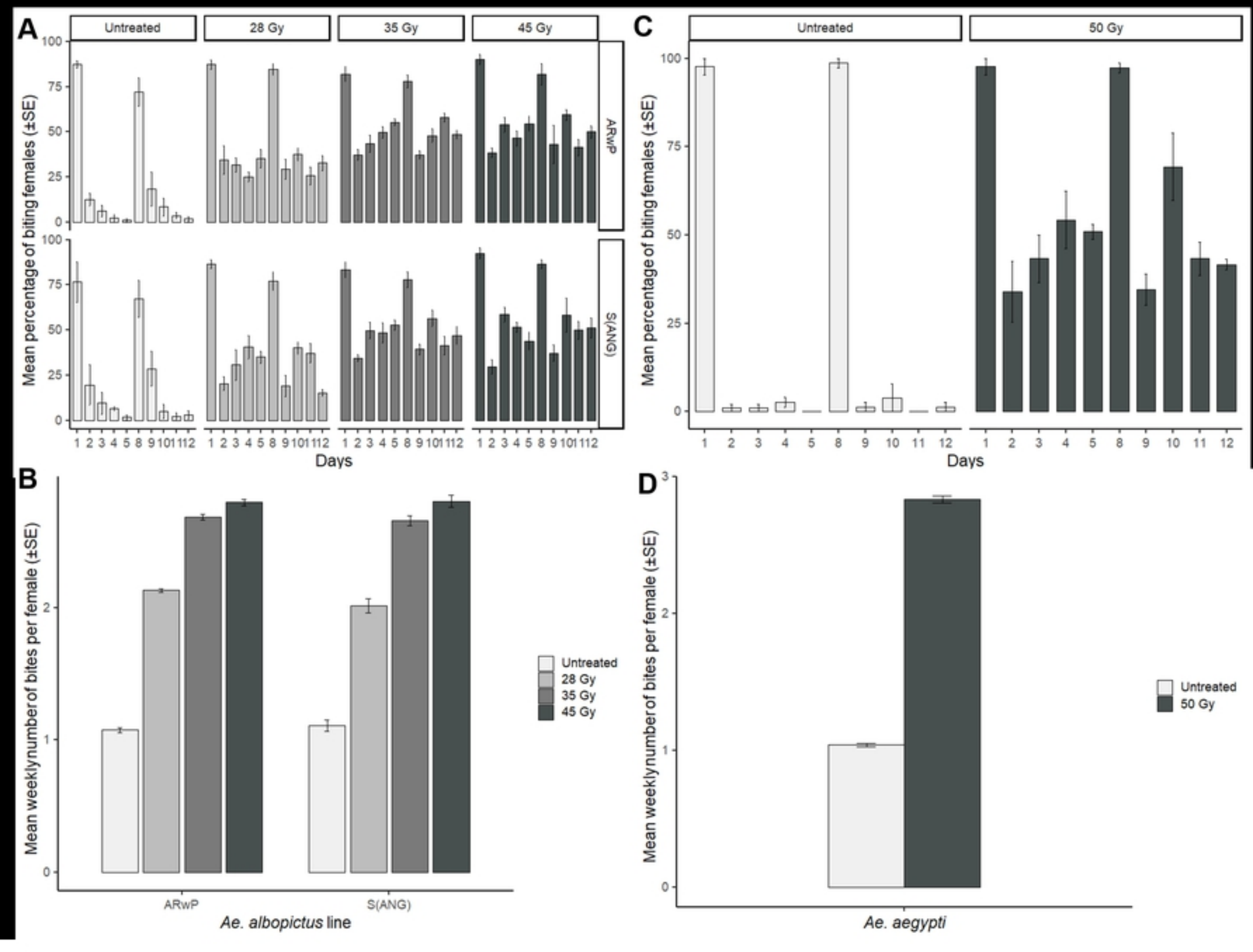
A
Untreated
28 Gy
35 Gy
45 Gy
ARwP
S(ANG)
Mean percentage of biting females (±SE)
Days
B
Mean weekly number of bites per female (±SE)
Untreated
28 Gy
35 Gy
45 Gy
ARwP
S(ANG)
Ae. albopictus line
C
Untreated
50 Gy
Mean percentage of biting females (±SE)
Days
D
Mean weekly number of bites per female (±SE)
Untreated
50 Gy
Ae. aegypti

Figure 2

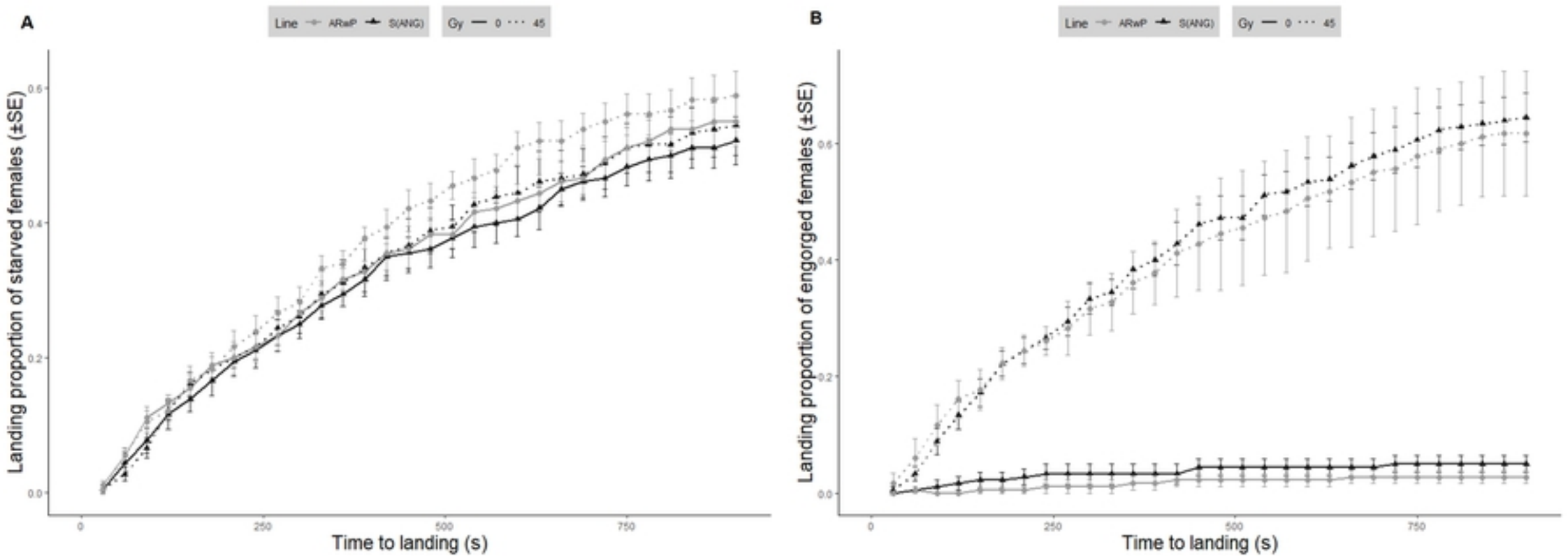
A
Line
ARwP
S(ANG)
Gy
0
45
Landing proportion of starved females (±SE)
0.0
0.2
0.4
0.6
0
250
500
750
Time to landing (s)
B
Line
ARwP
S(ANG)
Gy
0
45
Landing proportion of engorged females (±SE)
0.0
0.2
0.4
0.6
0
250
500
750
Time to landing (s)

Figure 3

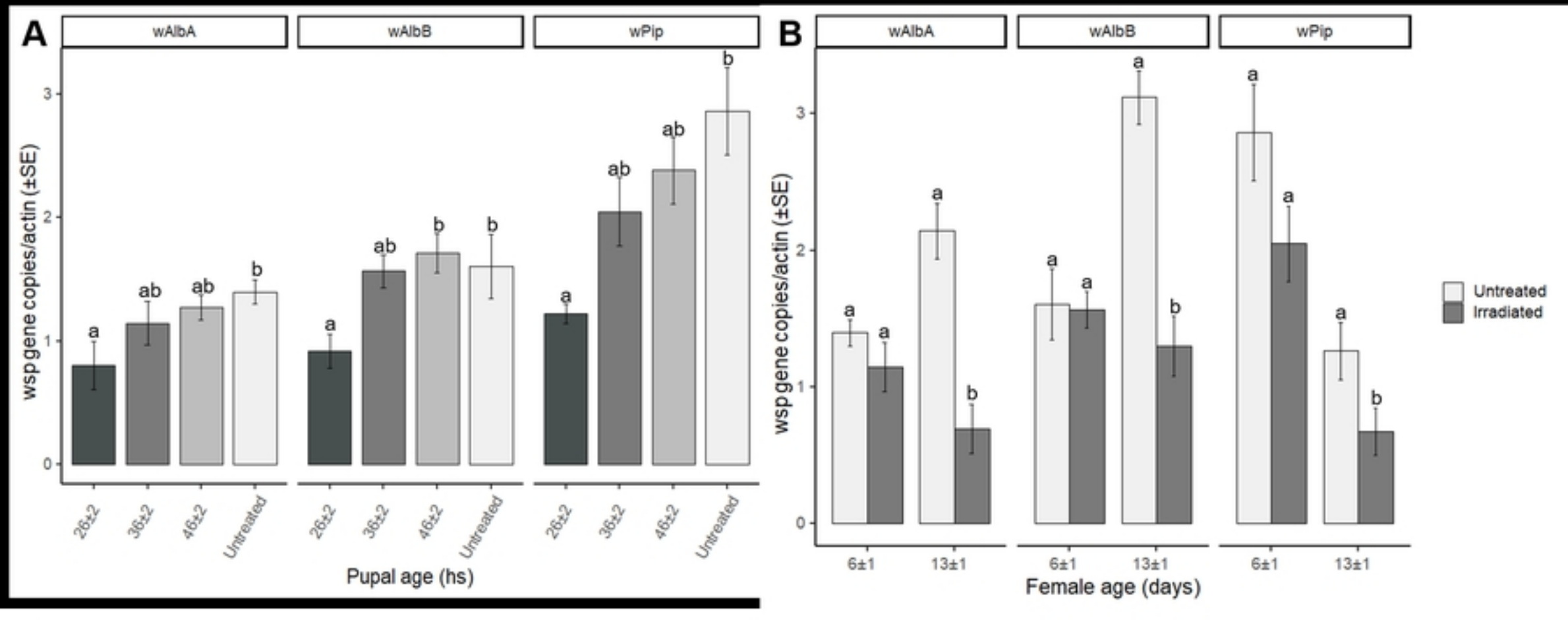
A
wAlbA
wAlbB
wPip
wsp gene copies/actin (±SE)
26±2
36±2
46±2
Untreated
Pupal age (hs)
B
wAlbA
wAlbB
wPip
wsp gene copies/actin (±SE)
6±1
13±1
Female age (days)
Untreated
Irradiated

Figure 4

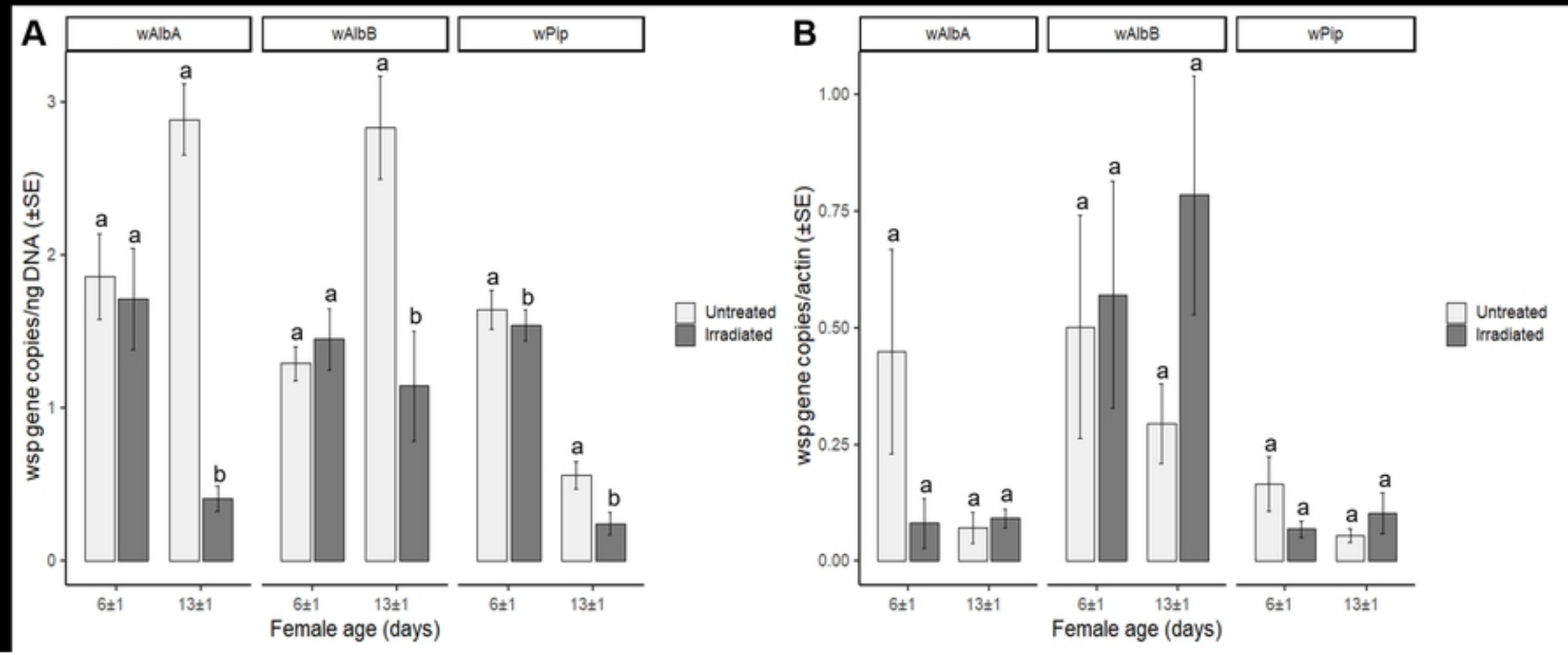
A
wAlbA
wAlbB
wPip
wsp gene copies/ng DNA (±SE)
Female age (days)
6±1
13±1
Untreated
Irradiated
B
wsp gene copies/actin (±SE)

Figure 5

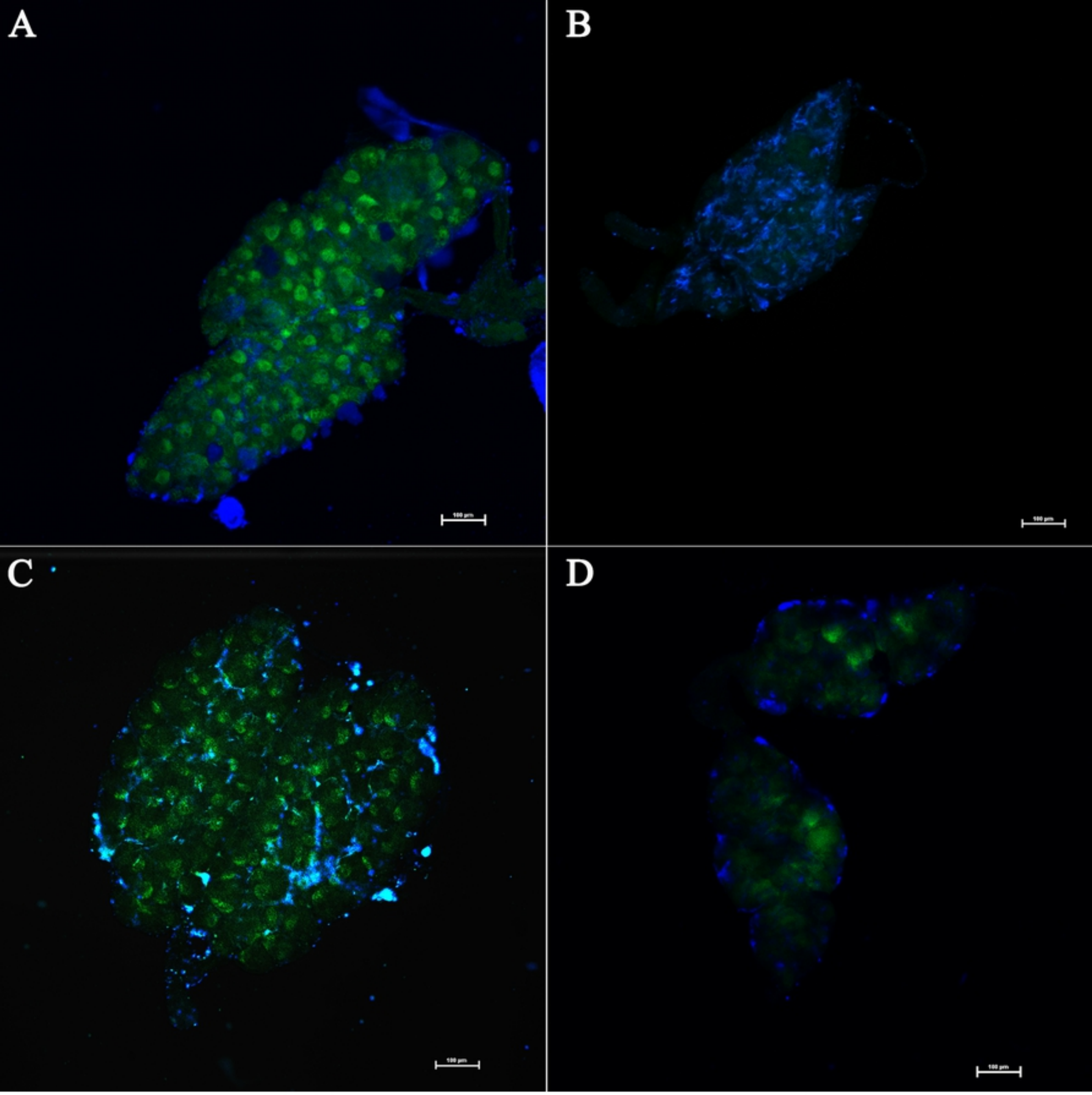

Figure 6