

GAMMA-IRRADIATION REDUCES SURVIVORSHIP, FEEDING BEHAVIOR, AND OVIPOSITION OF FEMALE *Aedes aegypti*

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ABSTRACT. *Aedes aegypti* is a prominent disease vector that is difficult to control through traditional integrated vector management due to its cryptic peridomestic immature-stage habitat and adult resting behavior, increasing resistance to pesticide formulations approved by the US Environmental Protection Agency, escalating deregistration of approved pesticides, and slow development of new effective chemical control measures. One novel method to control *Ae. aegypti* is the sterile insect technique (SIT) that leverages the mass release of irradiated (sterilized) males to overwhelm mate choice of natural populations of females. However, one potential liability of SIT is sex sorting errors prior to irradiation, resulting in accidental release of females. Our goal in this study was to test the extent to which irradiation affects female life-history parameters to assess the potential impacts of releasing irradiated females accidentally sorted with males. In this study, we determined that a radiation dose ≥ 30 Gy—a dose sufficient to sterilize males while preserving their mating competitiveness—may substantially impact longevity, bloodfeeding, oviposition, and egg hatch rate of female *Ae. aegypti* after being irradiated as pupae. These findings could reduce public concern for accidental release of females alongside irradiated males in an operational *Ae. aegypti* SIT control program.

KEY WORDS Biological control, operational vector control, pupa separator, yellow fever

INTRODUCTION

Aedes aegypti (L.) is a prominent disease vector (Morrison et al. 2008) that is extremely difficult to control through traditional integrated vector management due to: the cryptic peridomestic habitat exploited by this species for immature development and adult resting (Harwood et al. 2016); increasing resistance to pesticide formulations approved by US Environmental Protection Agency (Burkett et al. 2013); escalating deregistration of approved pesticides (Burkett et al. 2013); and slow development/registration of new effective chemical control measures (Avant 2012). Fortunately, the sterile insect technique (SIT), an approach proven successful for enduring control of other highly challenging insect species (Klassen and Curtis 2005), has been applied to the *Ae. aegypti* control problem in Indonesia (Ernawan et al. 2019), China (Zheng et al. 2019), experimentally in Mexico (Bond et al. 2019), and with a series of experiments and operational trials based in St. Augustine, FL (K. Linthicum, R. Xue, and D. Hahn, unpublished data), and Lee County, FL (R. Morreale and D. Hoel, personal communications). The SIT leverages the mass release of colony-reared males that are sterilized, by ionizing radiation or chemosterilants, to compete with wild females. Large numbers of sterile males are released into the field to bias the male mate options of females toward

those males that have been treated (Bourtzis et al. 2016). Females that mate with sterilized males produce infertile eggs due to genomic DNA damage in male sperm (Robinson 2005). Over time SIT can be used as one component of an integrated management program to reduce or even eliminate *Ae. aegypti* populations (Dame et al. 2009).

Unfortunately, one potential liability of SIT is inaccurate sex sorting of pupae prior to irradiation, resulting in an occasional release of females that may affect the public palatability of the SIT program by an increase in the number of *Ae. aegypti* females in the environment that could potentially bite humans and become infected by viruses (Dame et al. 2009) such as dengue, chikungunya, and Zika. However, little is known regarding the effect of radiation on female *Ae. aegypti* development and behavior given that historically greater emphasis has been placed on males instead of females for SIT programs. In this study we investigated impacts of gamma-radiation on bloodfeeding behavior, fecundity, fertility, and survivorship of female *Ae. aegypti* pupae to model potential outcomes for female pupae that might be accidentally included with male pupae after sex separation and before irradiation in an operational SIT program. We chose these parameters because they were important for determining the efficacy of radiation doses in a traditional SIT control program (WHO 2020).

MATERIALS AND METHODS

Experimental design

We carried out 2 nested sets of experiments to determine the effects that gamma-radiation had on the survivorship, bloodfeeding, oviposition, and egg

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hatch of *Ae. aegypti* (as determined by survivorship of larvae to the 3rd–4th instar after hatching from eggs of treated females). In experiment 1 (3 replicates) we evaluated a radiation dose range that spanned from 0 to 110 Gy. These dose values were loosely based on doses from Bond et al. (2019) in experiment 1. Experiment 2 (2 replicates) examined doses that ranged from 0 to 50 Gy. The doses selected for experiment 2 were based on the minimal values that achieved low fecundity and fertility in experiment 1. Specific target doses and absorbed doses are described in the Pupa Separation and Treatment subsection below.

Mosquito rearing and colony

Aedes aegypti were from the insecticide-susceptible Orlando (ORD) colony that had been maintained in a USDA–Agricultural Research Service (ARS) insectary since 1952. All adult mosquitoes used for evaluation were provided with 10% sucrose-soaked cotton ad libitum. Mosquitoes used in our experiments were not reintroduced into the primary colony. The colony and treatment replicates were bloodfed on mechanically defibrinated bovine blood in a lambskin condom (Trojan, Ewing, NJ) heated to approximately 30°C in a water bath. Bloodfeeding occurred throughout the day starting at 1000 h, with the blood being warmed periodically and feeding concluding at 1800 h. The ORD colony and all experiments were maintained at 27 ± 2°C and 30–70% RH, with a photoperiod of 12 h light and 12 h dark.

Aedes aegypti ORD colony maintenance was similar to that of Gerberg et al. (1994): eggs were volumetrically measured (0.3 ml) and hatched in a glass 10-ml scintillation vial filled with tap water. Eggs were added to rearing pans (14 in. × 19 in. × 2.5 in.) filled with 3 liters of room-temperature tap water and 50 ml of a prepared food slurry made up of 1,800 ml of water mixed with bovine liver powder and brewer's yeast (3:2 ratio; 80 g total). The pan and its contents were rocked to uniformly distribute the eggs and the food slurry. Pans were given 50 ml of the food slurry once every other day for a total of 3 feedings and rocked after every feeding. Seven days after hatching, pupae were ready to be collected through sieving. Adult mosquitoes were maintained in 30 × 30 × 30-cm cube cages (BugDorm-1; MegaView, Taichung, Taiwan). Mosquitoes were observed every 24–48 h, and dead adult mosquitoes were collected, sexed, and recorded. Dead adults were also recorded from the emergence cup, oviposition cup, oviposition paper, as well as the sucrose-soaked cotton balls, and these dead mosquitoes were added to the mortality count when they were removed from the cage.

Following the removal of the emergence cup, adult *Ae. aegypti* were given a chance to bloodfeed on mechanically defibrinated bovine blood in a lambskin condom for 30–60 min every 2–5 days. The number

of mosquitoes that fed on blood was observed and recorded within 30–60 min of the blood meal being placed into the cage.

Gravid *Ae. aegypti* were provided a black 4-oz soufflé cup (Solo Cup Co., Lake Forest, IL) filled ½ to ¾ with tap water and a 7.62 × 7.62-cm square of heavy stock (76 lb) seed germination paper (Anchor Paper, St. Paul, MN) 1–2 days following their initial bloodfeeding. Oviposition papers and cups were kept in the cage for 6 days and replaced on the 7th day.

Oviposition papers were allowed to dry for at least 48 h in an incubator set at 28 ± 2°C and 50–80% RH (Percival Scientific, Ames, IA). The papers were then individually sealed in labeled 1-gal bags (Ziploc; Dow, Midland, MI). After the oviposition papers were collected, they were scanned to 1,200 dpi using a tabletop scanner (9100-F; Canon Inc., Tokyo, Japan). Papers were left to sit for at least 7 days to allow the eggs to embryonate. The scanned papers were then cut with scissors; one cut portion of oviposition paper was removed from the storage bag and allowed to hatch in covered pans (1426A; Bioquip, Rancho Domingo, CA) with 300 ml of tap water provided with 3 ml of the liver powder and yeast slurry for 4 days. Following the 4 days pans were frozen in a –80°C freezer (SoLow, Cincinnati, OH). The pans were later thawed, and the dead larvae and pupae were counted to determine the number that survived, taking care not to count pupal or larval skins. The remaining portion of oviposition paper that was not allowed to hatch was scanned a 2nd time, and the difference in egg counts from the precut and the postcut papers was the number of eggs that were allowed to hatch. The original precut paper was the number of eggs collected.

Mosquito eggs were counted by eye from the digital scans of the oviposition paper using ImageJ (ver. 1.8.0_112; National Institutes of Health) (Rasband 2020). The multipoint tool, a feature of ImageJ, was used to mark every egg on the scanned image, then that number was recorded.

Pupa separation and treatment

Pupae (1–36 h) were sieved from the rearing pans (No. 20 brass sieve; 850 µm) and were separated by sex using a glass-wedge pupa separator (model 5412; John Hock Co., Gainesville, FL). The sexed pupae were counted into cohorts of 50 and distributed into petri dishes (60 × 15 mm) lined with filter paper using a modified/cut Pasteur pipette (approximately 3 ml) to accommodate the size of the pupae. The separated pupae were checked under a dissecting stereomicroscope to ensure that all pupae in the petri dish were alive, the correct sex, and the correct number. Excess water was removed from the petri dishes using a Pasteur pipette to keep the filter paper uniformly moist but ensure that pupae were not in standing water.

The prepared petri dishes were secured with masking tape, and the entire dish was treated with gamma-radiation generated from a Gammacell 1000 ^{137}Cs irradiator (GC45; Ottawa, ON, Canada) at a dose rate of 8.8 Gy/min; thus, higher doses required longer exposure. The HD-V2 film dosimeters (1 cm \times 1 cm, uncertainly below 2%; Ashland, Covington, KY) were individually placed in small paper envelopes and affixed on both the top and bottom of each petri dish. The film dosimeters were read by a DoseReader 4 spectrophotometer (Radiation General Ltd., Budapest, Hungary) with set at 590-nm Amber light approximately 24 h after irradiation. Alanine pellet dosimeters (Lot T030901; Far West Technology Inc., Goeta, CA; uncertainty 3.5%) were also used along with the film dosimeters side by side in some randomly chosen treatments to confirm the absorbed dose. Alanine pellets were read later at the National Center for Electron Beam Research, Texas A&M, College Station, TX. The target radiation doses used in experiment 1 were 0, 30, 50, 65, 85, 100, and 110 Gy. The target radiation doses used in experiment 2 were 0, 10, 20, 30, 40, and 50 Gy. The actual dose range received for each target dose was 10–11 Gy, 20–22 Gy, 31–33 Gy, 42–42 Gy, 49–51 Gy, 64–65 Gy, 83–84 Gy, 96–100 Gy, and 105–108 Gy. After irradiation, pupae were transported back to the laboratory in petri dishes. Using a squeeze bottle, pupae for each dose were rinsed into a 4-oz soufflé cup with their untreated male cohorts. In total, 50 untreated male pupae and 50 treated or control female pupae were combined into the 4-oz soufflé cup and allowed to emerge into a 30 \times 30 \times 30-cm cube cage (BugDorm). Pupae were allowed to emerge over 3 nights. Pupae that did not emerge and adults that died on the surface of the water were counted and sexed under a dissecting stereomicroscope.

Statistical analysis

All data were analyzed with R (R Core Team 2018). The effect of radiation dose on pupa to adult emergence was analyzed by a generalized linear model (GLM) with quasi-binomial family error distribution and radiation dose as a categorical fixed factor. Female bloodfeeding was analyzed by GLM with a quasi-binomial family error distribution, and radiation dose and bloodfeeding event number were treated as categorical fixed factors. Female fertility data were also analyzed by a GLM with a quasi-binomial family error distribution, with radiation dose and gonotrophic cycle treated as categorical fixed factors. Female fecundity in relation to radiation dose split by gonotrophic cycle was analyzed by ANOVA; radiation doses that eliminated egg production were not included in the analyses because of lack of variance. Adult longevity data were analyzed by a Cox regression model (Therneau and Grambsch 2000) with the library *survival* (Therneau 2015).

RESULTS

Experiment 1 included a radiation dose range from 0 to 110 Gy (0, 30, 50, 65, 85, 100, and 110 Gy). In this experiment, there was no statistically detectable effect of pupa radiation dose on the proportion of adults successfully emerging (Fig. 1A; GLM: radiation effect: $df = 6$, $\chi^2 = 7.54$, $P = 0.28$). Females treated at all doses >50 Gy had significantly shorter lives than nonirradiated controls (Fig. 2A; Cox regression, effect of dose: $df = 6$, $\chi^2 = 314.44$, $P < 0.001$), and mortality increased in a dose-dependent manner (Pearson's correlation for mortality at 8 days old, $r = 0.64$, $df = 19$, $P = 0.002$). Radiation exposure had clear effects on the proportion of females in a cage engaging in bloodfeeding across 3 events of exposure to a blood source (Fig. 3A; GLM quasi-binomial, effect of dose: $df = 6$, $\chi^2 = 116.83$, $P < 0.001$, effect of bloodfeeding event: $df = 6$, $\chi^2 = 2$, $P = 0.07 > 0.05$). When breaking down the effects of radiation dose across bloodfeeding events using post hoc contrasts, females receiving any radiation had reduced proportions of females feeding in the 1st and 3rd bloodfeeding events, but in the 2nd bloodfeeding event only treatments irradiated at >85 Gy had a reduced proportion of females bloodfeeding compared with nonirradiated controls (Fig. 3A).

In experiment 1 using a dose range from 0 to 110 Gy, female fecundity was reduced by all doses of radiation (Fig. 4A; ANOVA, effect of dose: $df = 6$, $F = 9.67$, $P < 0.001$; effect of gonotrophic cycle: $df = 1$, $F = 1.78$, $P = 0.20 > 0.05$). In fact, no females laid any eggs at all in many of the replicate cages across treatments (Table 1). There was some egg hatch in a few of the irradiated treatments (0–34), with one replicate cage having viable eggs in high doses in the 1st gonotrophic cycle (Fig. 5A and Table 1), but none of the females from irradiated treatments produced eggs that hatched in the 2nd gonotrophic cycle.

Experiment 2 included a narrower radiation dose range from 0 to 50 Gy. In experiment 2, there was also no effect of radiation dose applied to pupae on adult emergence (Fig. 1B; GLM: radiation effect: $df = 5$, $\chi^2 = 6.57$, $P = 0.26$). Females treated at all doses >10 Gy lived significantly shorter than nonirradiated controls, and the strongest mortality effects were at 40 and 50 Gy (Fig. 2B; Cox regression, effect of dose: $df = 5$, $\chi^2 = 59.07$, $P < 0.001$). In contrast to the previous experiment, irradiation had no detectable effect on the proportion of females observed bloodfeeding over the course of 5 events (Fig. 3B; GLM quasi-binomial, effect of dose: $df = 5$, $\chi^2 = 5.98$, $P = 0.308 > 0.05$).

In experiment 2, using a dose range from 0 to 50 Gy, female fecundity was strongly reduced by radiation doses >20 Gy (Fig. 4B; ANOVA, $df = 5$, $F = 17.18$, $P < 0.05$). In fact, no females laid any eggs at all in most replicates treated at >20 Gy. In those treatments that did produce eggs, a portion of the eggs laid were dried and exposed to hatching cues to assess fertility. Females treated as pupae at 20 Gy always had lower egg hatching than nonirradiated (0

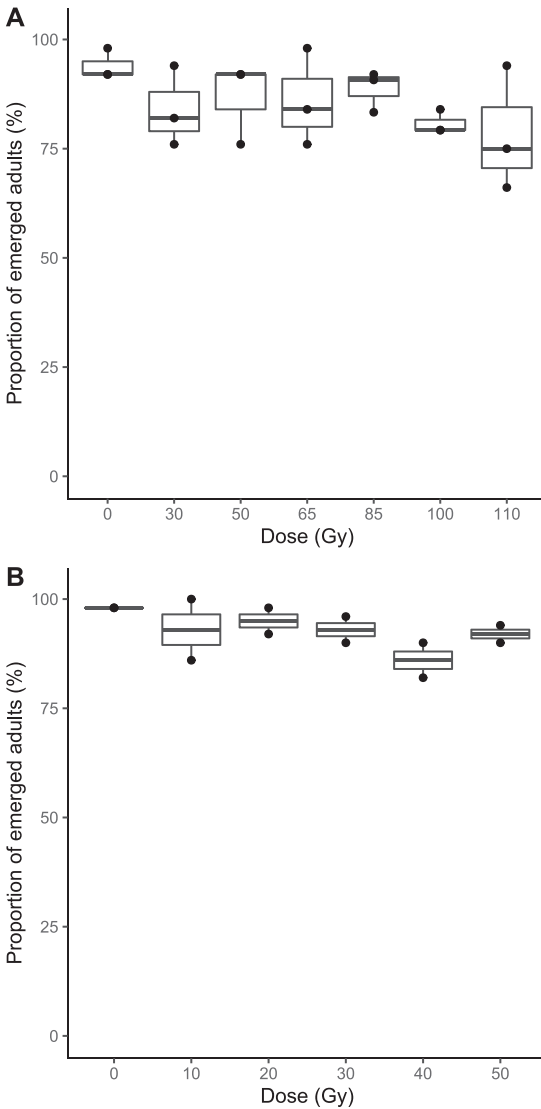


Fig. 1. (A) Adult emergence of irradiated female *Aedes aegypti* pupae was not affected by the radiation dose applied to pupae from experiment 1 that assessed doses 0–110 Gy (generalized linear model [GLM], radiation effect: $df = 6$, $\chi^2 = 7.54$, $P = 0.28$). (B) Adult emergence of irradiated female *Ae. aegypti* pupae was not affected by the radiation dose applied to pupae from experiment 2 that assessed doses 0–50 Gy (GLM, radiation effect: $df = 5$, $\chi^2 = 6.57$, $P = 0.26$).

Gy) females (Fig. 5B; GLM quasi-binomial, $\chi^2 = 49.82$, $df = 2$, $P < 0.001$). The one egg laid by a single replicate cage in the 30-Gy treatment group during the 2nd gonotrophic cycle did not hatch.

DISCUSSION

In this study, we investigated the effects of radiation on female *Ae. aegypti* pupal emergence,

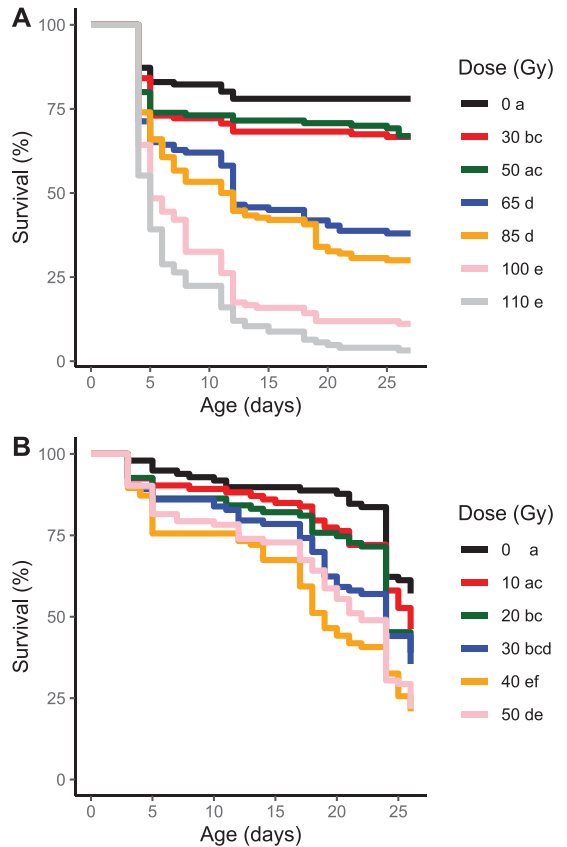


Fig. 2. (A) Adult longevity of *Aedes aegypti* females decreased at radiation doses >65 Gy applied to the pupal stage in experiment 1. Different letters show groups that are significantly different from each other (Cox proportional hazards regression, effect of dose: $df = 6$, $\chi^2 = 314.44$, $P < 0.001$). (B) Adult longevity of *Ae. aegypti* females similarly decreased compared to controls at radiation doses ≥ 20 Gy applied to the pupal stage in experiment 2. Different letters show groups that are significantly different from each other (Cox proportional hazards regression, effect of dose: $df = 5$, $\chi^2 = 59.07$, $P < 0.001$).

adult longevity, bloodfeeding, fecundity, and hatch rate. Specifically, we tested the extent to which a dose of radiation sufficient to sterilize male *Ae. aegypti* pupae for an operational SIT program could also reduce or eliminate the risks associated with inadvertent release of female *Ae. aegypti* (e.g., resulting from sex sorting errors in the lab). We exposed colony-reared female pupae to a structured array of radiation doses and examined a series of life-history traits relevant to the perceived risks of accidental adult female release (survivorship, blood-feeding, fecundity, and egg hatching). Similar to Bond et al. (2019), we found that female *Ae. aegypti* pupae were more sensitive to radiation than their male cohorts and that the minimum dose of radiation (approximately 30 Gy) needed to sterilize them was lower than the dose to effectively sterilize males

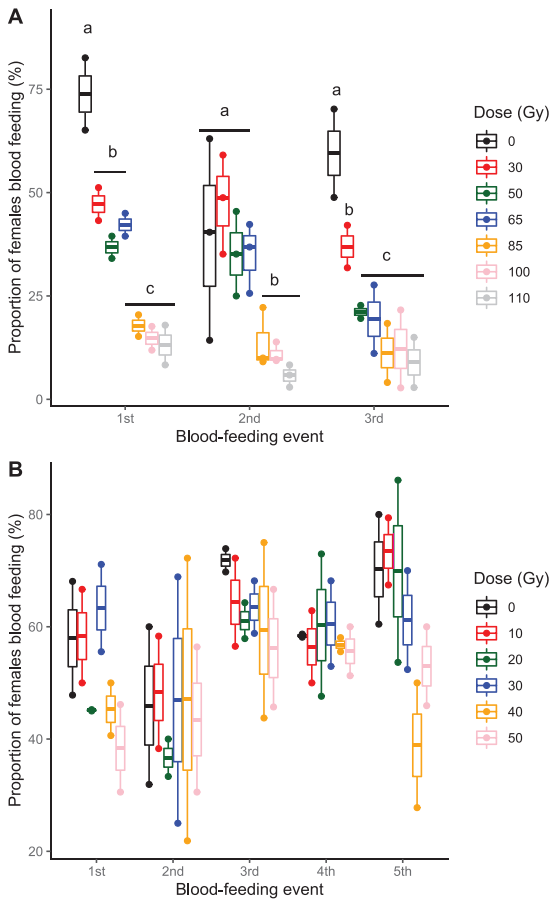


Fig. 3. (A) Radiation affected the proportion of *Aedes aegypti* females taking blood meals, with effects most pronounced in the 1st and 3rd bloodfeeding events in experiment 1. Letters denote groups that are significantly different from each other (generalized linear model [GLM] quasi-binomial, effect of dose: $df = 6$, $\chi^2 = 116.83$, $P < 0.001$; effect of bloodfeeding event: $df = 6$, $\chi^2 = 2$, $P = 0.07 > 0.05$). (B) In experiment 2, there was no detectable effect of radiation on the proportion of *Ae. aegypti* females taking blood meals over 5 events, although a greater proportion of females took blood meals in some events than others (GLM quasi-binomial, effect of dose: $df = 5$, $\chi^2 = 5.98$, $P = 0.308$; effect of bloodfeeding event: $df = 4$, $\chi^2 = 12.5$, $P = 0.01$).

while maintaining their competitiveness (K. Linthicum, D. Hahn, R. Xue, unpublished data). Therefore, based on the lower incidences of bloodfeeding and lower production of offspring in females irradiated at >30 Gy, it is evident that incidental release of female pupae sterilized alongside male pupae used for SIT pose a diminished threat in a male *Ae. aegypti* SIT program.

Survivorship of irradiated female *Ae. aegypti* differed across treatment doses in both experiment 1 and experiment 2 (Fig. 2). We would speculate that because survivorship at 30 Gy is lower than survivorship at 50 Gy, it would have similar, but

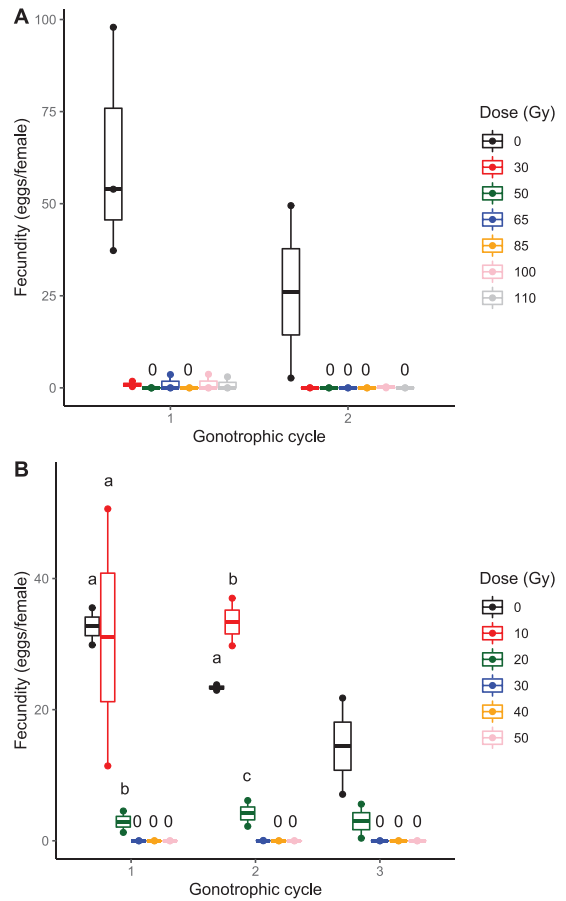


Fig. 4. (A) Exposure to doses of radiation >20 Gy decreased fecundity of *Aedes aegypti* females compared with controls in experiment 1 (ANOVA, effect of dose: $df = 6$, $F = 9.67$, $P < 0.001$; effect of gonotrophic cycle: $df = 1$, $F = 1.78$, $P = 0.20 > 0.05$). (B) In experiment 2, radiation had a substantial effect on the number of eggs female *Ae. aegypti* laid, with only one cage producing eggs at the 30-Gy dose in gonotrophic cycle 2 ($n = 1$ egg). No cages produced eggs at the 40- or 50-Gy doses in any gonotrophic cycle (ANOVA, $df = 5$, $F = 17.18$, $P < 0.05$). Letters denote post hoc separations using Tukey's honestly significant difference test, and treatments marked with a zero had no eggs produced in either replicate cage.

lower mortality than the 50-Gy dose. Treatment doses above 50 Gy diverged from that of the control dose (Fig. 2A). In experiment 2, survivorship differed from that of experiment 1 for the 30-Gy and 50-Gy treatment doses (Fig. 2). There was a significant difference in doses >10 Gy, with 30 Gy and 50 Gy being significantly different than the control dose (Fig. 2B). The 50-Gy dose of radiation appeared to have accelerated mortality compared with the 0-Gy dose group in experiment 2, unlike experiment 1. However, fewer replicates were completed for experiment 2, and these discrepancies may be less pronounced with more samples.

Table 1. Descriptive statistic table of eggs oviposited, larvae hatched, and hatch proportion separated by radiation dose across 2 experimental sets of irradiated *Aedes aegypti* Orlando colony females paired with nonirradiated males.

Dose (Gy)	Egg count				Larval count				Hatch proportion			
	Range	Mean	SEM	<i>n</i>	Range	Mean	SEM	<i>n</i>	<i>P</i> range	Mean <i>P</i>	SEM	<i>n</i>
Experiment 1												
0	104–3,916	1,480.5	400.09	10	0–1,218	294.1	122.07	10	0–0.88	0.47	0.11	10
30	0–79	16.4	7.78	10	0–11	1.3	1.09	10	0–0.2	0.04	0.02	10
50	0–1	0.1	0.1	10	0–0	0	0	10	0–0	0	0	10
65	0–101	15.2	10.75	10	0–34	5.6	3.72	10	0–0.79	0.14	0.09	10
85	0–20	2.1	1.99	10	0–13	1.3	1.3	10	0–1.18	0.12	0.12	10
100	0–70	7.3	6.97	10	0–3	0.3	0.3	10	0–0.06	0.01	0.01	10
110	0–57	6.44	6.32	9	0–3	0.3	0.3	9	0–0.07	0.01	0.01	9
Experiment 2												
0	319–1,706	1,081.67	190.83	6	163–478	324	45.49	6	0.4–0.89	0.74	0.07	6
10	490–2,430	1,242	300.23	6	99–711	464.67	97.09	6	0.38–2.14	0.99	0.33	6
20	15–258	140.17	40.2	6	1–39	22.23	5.69	6	0.09–0.41	0.29	0.05	6
30	0–2	0.33	0.33	6	0–0	0	0	6	0–0	0	0	6
40	0–0	0	0	6	0–1	0.17	0.17	6	0–0	0	0	6
50	0–0	0	0	6	0–0	0	0	6	0–0	0	0	6

Bloodfeeding observed across the 2 experiments differed as visualized by means separated by dose and bloodfeeding event in Fig. 3. The lack of differences between doses observed in experiment 2 (Fig. 3B) compared with experiment 1 (Fig. 3A) are probably due to the finer scale of the doses applied and the low power due to the low sample size. However, it was observed that the bloodfeeding interval (“event”) was significantly different regardless of the dose of radiation applied. This may in part be due to the close chronological proximity of bloodfeeding opportunities. Although the 1st blood meal was approximately 4 days after adult emergence, the 2nd blood meal was only 2 days after the 1st blood meal, thus leading to a reduced number of freshly fed females observed, because females may still be digesting their 1st meal and therefore have no interest in a 2nd meal. The 3rd bloodfeeding was at least 3 days following the 2nd blood meal, but also after they had been given an opportunity to oviposit.

A cooperative follow-up study to investigate bloodfeeding habit by USDA–Center for Medical, Agricultural, and Veterinary Entomology (CMAVE) and the Anastasia Mosquito Control District, St. Augustine, FL, demonstrated that if bloodfeeding opportunity is limited to only 15 min, irradiated females generally prefer not to take a blood meal (Cunningham et al. 2020). Although it is not the only tissue damaged by radiation exposure, we suspect that damage to the female mosquito gonad tissue, as observed in Guthrie and Brust (1971) and Ali and Rozeboom (1972), may reduce female host-seeking and bloodfeeding behavior. Furthermore, we suspect that greater radiation exposure leads to increased damage to gonad tissue and thus a greater reduction in bloodfeeding habit. Furthermore, radiation has previously been shown to damage the midguts of other insects (Calkins and Parker 2005), and it is thus possible that damage to the midgut could also affect female motivation to feed. More work is needed to

examine the effects of radiation on female bloodfeeding and digestion with regard to behavior, vector capacity, and disease transmission and testing the extent to which irradiating females leads to lower biting probabilities in field and laboratory environments.

Our results documented that egg production of irradiated female *Ae. aegypti* could be significantly reduced based on the dose of radiation applied to them as pupae (Fig. 4). Terzian and Stahler (1958) showed that egg production of irradiated adult female *Ae. aegypti* could be significantly reduced by exposure to radiation (22 Gy), with no eggs being produced after 8 days in doses higher than 43 Gy. McCray et al. (1961) described that both male and female *Ae. aegypti* pupae were not only sterilized by exposure to 90–150 Gy, but that the females simply did not oviposit. Asman and Rai (1972) described how sensitive female gonads of *Ae. aegypti* are to radiation compared with male gonads, and observed female adults were completely infecund when irradiated at doses ≥ 35 Gy as pupae. Kittayapong et al. (2018) demonstrated that a dose of 50 Gy to female pupae was enough to render female adult *Ae. aegypti* completely sterile, producing no eggs. In 2019, Bond et al. (2019) described complete loss of egg production from females irradiated at doses ≥ 30 Gy. These results match our own, where few or no eggs were generated from female *Ae. aegypti* pupae exposed to doses ≥ 30 Gy.

Radiation applied to female mosquitoes had a similar effect on oviposition behavior across species. For instance, *Anopheles quadrimaculatus* Say female pupae exposed to 103 Gy combined with nonirradiated males produced no eggs (Davis et al. 1959). *Anopheles arabiensis* Patton pupae exposed to 70 Gy of ionizing gamma-radiation were rendered sterile and produced no eggs (Dandalo et al. 2017). Similarly, observations by Abdel-Malek et al. (1966) demonstrated that adults from irradiated

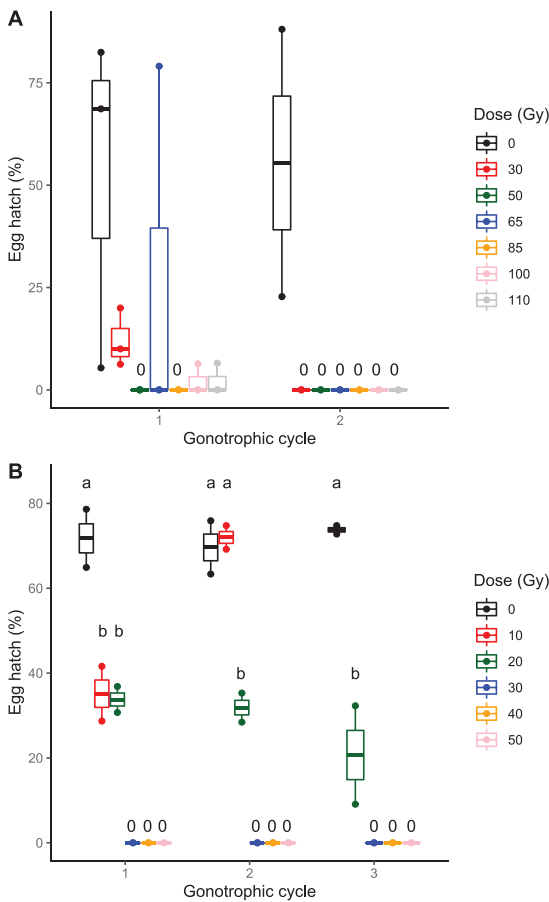


Fig. 5. (A) Radiation had an effect on the proportion of eggs that hatched in the 1st gonotrophic cycle from irradiated female *Aedes aegypti* in experiment 1, although surprisingly there were some eggs that hatched from cages of females irradiated at very high doses even though eggs from lower doses did not hatch (generalized linear model [GLM] quasi-binomial, $df = 1$, $\chi^2 = 25.5$, $P < 0.001$). Egg hatching could only be analyzed from the 1st gonotrophic cycle because no eggs were laid by cages of irradiated females (30–110 Gy) in the 2nd gonotrophic cycle (represented by zeros in the figure). (B) Radiation had an effect on the proportion of eggs that hatched from irradiated female *Ae. aegypti* in experiment 2 (GLM quasi-binomial, $df = 2$, $\chi^2 = 49.82$, $P < 0.001$). Egg hatching could only be analyzed for the 0-, 10-, and 20-Gy doses because treatments exposed to 30 Gy or above had either no eggs laid, or the few eggs produced did not hatch (represented by zeros in the figure).

female *An. pharoensis* Theobald pupae oviposited significantly fewer eggs than controls at 17 Gy. *Culex pipiens quinquefasciatus* Say exposed to 52 Gy as pupae produced eggs in doublets or triplets lacking an egg raft structure; however, at 61 Gy females did not produce any eggs (Smittle 1968). *Aedes polynesiensis* Marks female “early” pupae were exposed to 40 Gy of radiation and were rendered completely sterile and did not oviposit any eggs; however,

irradiated “late” pupae exposure produced significantly fewer eggs than the controls (Brelsfoard et al. 2009). Balestrino et al. (2010) documented that irradiating female *Ae. albopictus* (Skuse) rendered them completely sterile at ≥ 30 Gy, producing no eggs. In general, based on scientific literature and our results, female mosquitoes exposed to radiation will be completely sterilized at or below doses that will effectively sterilize their male cohorts.

Our observations on fecundity of female *Ae. aegypti* exposed to radiation support the findings of many others. First, we found that radiation not only sterilizes female *Ae. aegypti* when treated in the pupal stage, but it works to reduce fertility by reducing the number of eggs generated. Second, we observed that the dose of radiation to sterilize female *Ae. aegypti* pupae is ≥ 30 Gy as observed by Balestrino et al. (2010) in *Ae. albopictus* and similar to that observed by Asman and Rai (1972) 35 Gy in *Ae. aegypti*. This threshold is useful because as suggested by Curtis (1976) and supported by Brelsfoard et al. (2009), females infected with *Wolbachia* for an incompatible insect technique (IIT) program could be sterilized using radiation in a joint IIT–SIT operation such as that implemented successfully by Zheng et al. (2019) to eliminate a mosquito population. The purpose of irradiating *Wolbachia*-infected male *Ae. aegypti* was so that any lingering females that are not removed from the released males are sterilized (Zheng et al. 2019). This ≥ 30 -Gy threshold marks the minimum dose necessary to sterilize female *Ae. aegypti* in order to implement a similar IIT–SIT joint operation to potentially reduce or eliminate an endemic population of *Ae. aegypti* (Fig. 5).

With regard to the anomalous fertility and fecundity data recorded from some of the replicate cages given high doses, we speculate that in the sex sorting process a fertile female was mistakenly mixed into the batch of nonirradiated males. Although this did not happen every time and in every treatment, the presence of 1 fertile (i.e., nonirradiated) female would compromise the observed declining or zero reproduction at a dose. To confirm our speculation, we conducted a pilot study that intentionally released a fertile female in with females dosed at 0, 50, and 110 Gy. The results were similar to those seen from cages that diverged from the declining trend in egg number and larval hatch (data not shown).

In conclusion, the maintaining of a successful SIT program for *Ae. aegypti* will present a number of challenges to produce, separate, irradiate, and release competitive sterile males. Although recently developed technological advances seemingly can achieve near-perfect elimination of females from males in SIT workflows (Crawford et al. 2020), not all programs may have access to this technology due to cost or other limitations. The SIT programs may have to rely on sexual dimorphism to separate males from females (Christophers 1960). The significant difference in pupal size diminishes if immatures are

fed less, or develop smaller than pupae from normally fed larvae due to crowding conditions (Koenraadt 2014). To rear the vast numbers of pupae needed to release for control purposes it is necessary to rear massive numbers of larvae. Unfortunately, this can lead to errors in separation, leading to females ending up mixed in with male cohorts—therefore being released with males.

The public perception of vector control operations can make or break experimental studies and operational programs. In the USA, public reaction to experimental solutions and adopted practices influences the election of commissioners that control the budgets and employment of managing personnel of mosquito control districts. An increase in biting pressure due to accidental release of females would not function in favor of supporting experimental SIT operations, and support for researching/implementing an SIT program could quickly wane due to lack of public acceptance. Our findings can directly address public perception and assuage concerns related to incidental female release—with one caveat. Our results demonstrating diminished bloodfeeding suggest that irradiated female *Ae. aegypti* that are accidentally released would have decreased vectorial capacity; however, additional work specifically on vector competence and other traits including dispersal and host-seeking behavior is needed to confirm that irradiated females have a reduced risk of disease transmission.

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