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Authors: Allinghi, Armando, Gramajo, Celia, Willink, Eduardo, and Vilardi, Juan

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INDUCTION OF STERILITY IN *ANASTREPHA FRATERCULUS* (DIPTERA: TEPHRITIDAE) BY GAMMA RADIATION

ARMANDO ALLINGHI[1], CELIA GRAMAJO[2], EDUARDO WILLINK[2] AND JUAN VILARDI[3]
[1]Comisión Nacional de Energía Atómica, CNEA

[2]Estación Experimental Agro-industrial Obispo Columbres, Tucumán, Argentina

[3]Depto. Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales
Universidad Buenos Aires. (1428) Buenos Aires, Argentina

ABSTRACT

In relation to the application of the sterile insect technique (SIT) for the South American fruit fly *Anastrepha fraterculus* (Wiedemann), we analyzed the effect on adult fertility of different doses of gamma irradiation and the age of pupae at the time of irradiation. In a first experiment, we applied doses of 50, 70, and 90 Gy to pupae at 24, 48, 72, and 96 h before adult emergence. In a second experiment we irradiated pupae 48 h before emergence with 20, 40, and 60 Gy and estimated male and female fertility and sperm transfer by irradiated males. The results indicated pupal age at irradiation does not significantly affect male fertility. If males irradiated with 60 Gy are crossed to non-irradiated females the fertility is about 1%. Females irradiated with 40 Gy did not lay eggs independently of the male to which they mated. No significant effects of radiation were observed with respect to the ability of males to transfer sperm. A dose of 70 Gy applied 48 h before adult emergence induces 100% sterility in both males and females.

Key Words: SIT, South American fruit fly, fertility, sperm transfer, sterility, pupal age

RESUMEN

Para la aplicación de la técnica del insecto estéril (TIE) en *Anastrepha fraterculus* (Wiedemann), en este trabajo analizamos el efecto de diferentes dosis de irradiación gamma y la edad óptima de la pupa al momento de la irradiación. En el primer experimento se evaluaron las dosis de 50, 70, y 90 Gy en pupas de 24, 48, 72, y 96 h antes de la emergencia del adulto. En el segundo experimento se irradiaron pupas 48 h antes de la emergencia con dosis de 20, 40, 60 Gy y se estimó la fertilidad de los machos y las hembras, y la transferencia de espermas por los machos irradiados. Los resultados indicaron que la irradiación no modificó significativamente la fertilidad de los machos. En las cruzas de machos irradiados a 60 Gy con hembras no irradiadas se observó 1% de eclosión larvaria, mientras que las hembras irradiadas a 40 Gy no pusieron huevos. La irradiación no afectó significativamente la transferencia de espermas de los machos tratados. Por lo tanto, una dosis de 70 Gy aplicada 48 h antes de la emergencia del adulto induce 100% de esterilidad tanto en machos como en hembras.

Translation provided by the authors.

The South American fruit fly *Anastrepha fraterculus* (Wiedemann) (Diptera: Tephritidae) is an important pest for fruit production in Argentina (Stone 1942). This species is native to the Americas, most probably South America, and is widely distributed throughout the tropical and subtropical regions (between the latitudes 27°N and 35°S). Its range includes southern USA (South Florida and Rio Grande Valley, Texas), Central America, Caribbean Islands, and South America, from Trinidad and Guyana to Central Argentina (Steck 1999; Aluja 1994; Hernández-Ortiz 1992).

There are at least 80 host species of *A. fraterculus*, including many economically important fruit species (Norrbom & Kim 1988). Tropical fruit flies not only cause great losses in fruit and vegetable production, but they also seriously impede international trade because of quarantine regulations (Klassen & Curtis 2005). In particular, the presence of *A. fraterculus* in the orchards reduces the possibility of exporting fruits and other horticultural products to the northern hemisphere (SENASA 1997). The export of fruits and vegetables to pest free areas or those that have implemented control programs against this pest requires the application of a quarantine treatment. Another common problem is that the intensive use of chemical insecticides is associated with environmental contamination. Furthermore, insects have been found to develop resistance to almost every chemical class of insecti-

cide (Brown & Payne 1988). This includes some tephritids, such as *Bactrocera oleae* (Gmelin) (Vontas et al. 2002) and *Bactrocera dorsalis* Hendel (Hsu et al. 2004).

Recent studies indicate that populations of *A. fraterculus* from Argentina and Southern Brazil are not differentiated genetically (Alberti et al. 2002) and that 4 populations from different regions of Argentina do not show reproductive isolation (Petit-Marty et al. 2004). These findings suggest that the sterile insect technique (SIT) might be applied successfully against *A. fraterculus* at least at a regional scale.

In other tephritids, such as *Ceratitis capitata* (Wiedemann), the irradiation process may reduce the mating performance of the sterilized males (Calcagno et al. 2002; Lux et al. 2002). An essential requirement for a successful SIT is the application of a sterilization protocol to mass reared insects that ensures sterility with a minimal detriment of the mating competitiveness and viability of the released insect. Germ cells (oocytes and spermatids) are highly radiosensitive and when exposed to ionizing radiation, dominant lethal mutations are induced (Muller 1927). The dominant lethal mutations produced by radiation in insects depend mainly on the dose, insect type, size, and sex (Hooper 1989). Radiosensitivity also depends on other factors such as irradiation temperature, humidity, ploidy level, mitotic cycle phase, and metabolic condition (Enkerlin et al. 1997).

In species of the genus *Anastrepha*, studies on the effect of pupal age and radiation dose on the induced sterility are not completely consistent. Rhode et al. (1961) reported that *Anastrepha ludens* (Loew) pupae irradiated 96 h before emergence with 40 Gy showed 100% male sterility. By contrast, according to Velasco & Enkerlin (1982), the dose needed to induce sterility in the same species should be much higher. They reported that 40 Gy and 100 Gy induced 90% and 99% sterility, respectively, when pupae were irradiated 72 h before emergence. In the case of *Anastrepha suspensa* (Loew), Burditt et al. (1975) irradiated pupae with 40 Gy at 48 h before emergence and observed complete adult sterility whereas Calkins et al. (1988) reported that lower irradiation doses (30 Gy) applied 24-48 h before emergence induced high levels of sterility.

The efficiency of sterilized insect release programs depends to a great extent on the ability of laboratory reared sterile males to mate with, and transfer sperm to, wild females in the field (McInnis 1993). Usually, immediately after copulation 90% of sperm is found in the spermathecae of the female (Yuval et al. 1996). Mossinson & Yuval (2003) have shown that females with fewer sperm in their spermathecae show a higher tendency to remate. Remating may reduce the efficiency of the SIT if the second mating occurs with a wild male. However, this is very unlikely as sterile males far outnumber wild males in an SIT programme.

We analyzed under laboratory conditions the effect of different doses of gamma irradiation and the age of pupae at the time of irradiation on the induced sterility and the ability to transfer sperm in *A. fraterculus*.

Materials and Methods

The *A. fraterculus* individuals studied were from a strain reared since 1997 at Estación Experimental Provincial Obispo Colombres, Tucumán, Argentina. Pupae were sent to Buenos Aires (Centro Atómico Ezeiza, Grupo Agronómico, CNEA) by surface and there were kept under controlled conditions (25 ± 1°C, 75 ± 5% RH, and a photoperiod of 12:12 (L:D). Adult diet was composed of white sugar: yeast (Calsa, S:A:, Tucuman, Argentina) (3:1). Water was provided as 1% agar in 12-mL vials. Food and water were changed each once a week.

Pupae were irradiated at the Centro Atómico Ezeiza facility (Comisión Nacional de Energía Atómica, Argentina) in a Gammacell 220 (MDS Nordion, Canada) irradiator, with ^{60}Co source (dose rate for the first and second experiment: 1.67 Gy min^{-1} and 1.60 Gy min^{-1}).

Experiment 1: Optimal Pupal Age for the Irradiation Treatment

Pupae were irradiated 24, 48, 72, or 96 h before adult emergence. In each case four different radiation doses were applied: 0 (control), 50, 70, and 90 Gy. Upon emergence adults were separated by sex and kept for 15 d under controlled conditions at 25°C, 80% RH, and a photoperiod of 13:11 (L:D), and light intensity of 3500 lux. At this age all individuals are sexually mature (De Lima et al. 1994). For each treatment, male fertility was evaluated by exposing ten fertile (non-irradiated) females to a sample of ten treated males for 20 d in a 3000-cm^3 flask.

After exposure to males, females were transferred to egg collecting flasks that were similar to those used for the crossing, but they had an artificial egg laying substrate hanging from the top. It consisted of 3-cm diameter sphere made of 3.5 g agar and 0.05 g red dye (color index 14700) dissolved in 300 mL water. The sphere was wrapped in Parafilm® (Boller 1968; Manso 1998). The artificial substrates were removed after an exposure period of 48 h to the inseminated females, The Parafilm® was removed, and the eggs were manually extracted and transferred to a wet Petri dish. Eggs were kept for 72 h at 25°C. After this period, the numbers of hatched and non-hatched eggs were recorded. The experiment was replicated 3 times for each treatment

Experiment 2: Optimal Dose for the Sterilization of Pupae 48 h before Adult Emergence

The effect of irradiation on male or female fertility was analyzed by mating flies irradiated with different doses of gamma rays with non-irradiated flies of the opposite sex. Pupae were treated 48 h before emergence with 0 (control), 20, 40, and 60 Gy. Emerged adults were kept under the same conditions as those of Experiment 1. Male fertility was evaluated in a similar way as that described for Experiment 1, but in this case 15 males and females were used. Six replicates were obtained for the 20, 40, and 60 Gy treatments. Female fertility was tested by exposing 15 irradiated females to 15 mature fertile males. In this case 2 replicates were obtained for each radiation dose. Four replicates of the crossing of fertile males and females were used as the control treatment for both male and female fertility tests.

The method of egg collecting was similar to that described for Experiment 1. In this case females were allowed to lay eggs for 1 month. During this period 7 egg collections (one every 3-5 days) were made for each treatment. A total of 28 samples were obtained for the control (7 collections × 4 replicates) and 14 for each group of irradiated females (7 collections × 2 replicates).

Sperm Transfer

In order to determine if sterile males are able to transfer sperm, spermathecae of fertile females mated to irradiated and non-irradiated males in Experiment 2 were observed. Females were sacrificed and fixed in 70% ethanol. Spermathecae were dissected on a paraffin wax layer with the help of entomological needles. Spermathecae were transferred onto a slide with a drop of acetic orcein (Guillén-Aguilar 1983), covered with a coverslip, and pressure applied with the thumb to break them and release sperm into the dyeing solution. The presence or absence of sperm was scored with a stereoscopic microscope at 100× magnification.

Statistical Methods

To determine the best time to irradiate pupae, the percent of egg hatch was compared among eggs laid by females inseminated by fertile males and males irradiated with 50, 70, and 90 Gy at 24, 48, 72, and 96 h before adult emergence by means of a homogeneity chi square test. To evaluate the effect of different radiation doses 48 h before adult emergence on male fertility, the percent of egg hatch was compared among eggs laid by fertile females inseminated by males treated with different radiation doses. The method used was non-parametric Kruskall-Wallis analysis of variance. Pair wise comparisons were performed by Mann-Whitney *U* test. The percentage of egg hatch was compared for each treatment among the 7 consecutive egg collections by means of Kruskall-Wallis analysis of variance.

Irradiated females tended to lay lower numbers of eggs and their eggs showed a reduced egg hatch. Because only 2 classes were able to lay eggs, i.e., control and females irradiated with 20 Gy, they were compared by means of Mann-Whitney *U* test. The proportion of spermathecae with or without sperm was compared by means of Fisher's exact test. All statistical tests were performed with the program STATISTICA, ver. 5.1 (StatSoft 2000).

Results

Optimal Pupal Age for Irradiation

The percent egg hatch of the control group (females inseminated with non-irradiated males) was about 87%. This values drops dramatically ($P = 0$) when the males were irradiated even with the lowest dose (50 Gy). The doses of 70 and 90 Gy induced total sterility independent of pupal age at the time of irradiation (Table 1). The comparison of percent egg hatch among ages for the treatment with 50 Gy indicated that the differences are not significant ($\chi^2 = 2.49, P = 0.93$). These results indicate that within the interval considered, pupal

TABLE 1. PERCENT OF EGG HATCH AND TOTAL NUMBER OF SCORED EGGS (IN PARENTHESES) OVIPOSITED BY FERTILE FEMALES INSEMINATED BY NON-IRRADIATED MALES (CONTROL) AND MALES IRRADIATED WITH DIFFERENT DOSES OF GAMMA RAYS AND AT DIFFERENT STAGES OF PUPAL DEVELOPMENT.

	Dose (Gy)			
Pupal age[1]	0 (Control)	50	70	90
24	—	0.56 (533)	0 (646)	0 (387)
48	—	0.32 (930)	0 (382)	0 (845)
72	—	0.18 (550)	0 (625)	0 (886)
96	—	0.51 (787)	0 (614)	0 (777)
--	86.98 (976)			

[1]Hours before emergence.

age at the time of irradiation does not affect the sterility induced by gamma radiation in males.

Evaluation of Optimal Dose for Pupal Irradiation

Radiation reduced male fertility as determined by egg hatch, from about 80% in the control group (0 Gy) to about 1% in the group treated with 60 Gy (Table 2). According to Kruskall-Wallis analysis of variance, the differences among treatments were highly significant (H = 123.08, $P \cong 0$). Pairwise comparisons by Mann-Whitney tests indicated that all pairs differ significantly (U= 0-362, Z = 4.7-7.6, $P < 10^{-5}$). The percentage of egg hatch did not differ significantly within treatments among the collections throughout the one-month period the females were allowed to oviposit (H = 7.97-11.08, P = 0.24-0.09).

In the case of females, the radiation treatment affected both the number of eggs laid and the percent hatch (Table 3). Females irradiated with 40-60 Gy were not able to lay eggs at all. Females irradiated with 20 Gy laid only a third of the eggs as compared with non-irradiated females. The differences between the control (0 Gy) and the treatment with 20 Gy were highly significant for both percent of egg hatch (U = 16, Z = -4.8, P = 1.6 10^{-6}) and number of eggs that were laid (U = 1.5, Z = -5.2, P = 2 10^{-7}).

Evaluation of Effects of Radiation on Sperm Transfer

The proportion of spermathecae containing sperm was slightly higher in females mated to fertile males (34/45) than in females mated to irradiated males (46/70) but these differences were not significant according to Fisher's exact test (P = 0.37).

Discussion

The efficient application of the SIT requires the precise determination of the optimal conditions for pupal irradiation. This question is very important to avoid undesirable side effects of the radiation treatment such as physiological or behavioral alterations that might reduce the competitiveness of irradiated males. Irradiation of larvae and young pupae may produce adult sterility but also extensive somatic damage with unwanted effects as aspermia or reduced adult survival. The irradiation of mature pupae has been shown to improve the field performance of mass reared and sterilized males (Hooper 1989). In most insects meiosis during spermatogenesis occurs before the last molt (Chapman 1998) and mature spermatozoa have already left the testis at the time of adult emergence from the pupa (Wigglesworth 1965). Radiation may induce high levels of dominant lethal mutations in spermatids and spermatozoids as well as the death of premeiotic cell stages and atrophy of germinal tissues. Usually, sterility is permanent, although in exceptional cases non-damaged spermatozoids may be regenerated from spermatogonia.

With respect to the best time to apply radiation treatment, studies on other tephritid species such as *B. dorsalis*, *Bactrocera cucurbitae* (Coquillett), *Bactrocera oleae* (Gmelin), *C. capitata*, *A. ludens*, *Anastrepha obliqua* (Macquart), and *A. suspensa* demonstrate that pupae irradiated 24-48 h before emergence exhibit high levels of sterility (Velasco & Enkerlin 1982; Hooper 1989; Walder & Calkins 1993; Toledo 1993).

Our results in *A. fraterculus* indicate no differences in the percentage of egg hatch among eggs produced by individuals irradiated at different ages within the interval 24-96 h before emergence. Therefore, the question remains as to what is optimal age within this period to produce sterile males with the best competitiveness. Although this aspect remains to be studied, we adopted the generalized criterion applied in SIT operations against other tephritid flies of irradiating 48 h before emergence (Velasco & Enkerlin 1982; Hooper 1989; Walder & Calkins 1993; Toledo 1993). As stated by Hooper (1989) irradiation at early developmental stages is highly detrimental due to the high metabolic activity and morphological changes during the metamorphosis process. If radiation is applied to mature pupae 24-48 h before

Table 2. Effect of dose of gamma irradiation on the percent of hatch of eggs laid by fertile females mated to males irradiated 48 h before emergence. n = number of eggs scored.

#Dose (Gy)	Egg hatch[1] (% ± SD)	n
0	80.7 ± 8.06 a	3384
20	9.8 ± 4.53 b	4760
40	3.0 ± 2.00 c	5042
60	1.3 ± 1.70 d	5175

[1]Different letters mean groups that differ statistically.

Table 3. Number of eggs collected from females treated with different doses of gamma rays and their corresponding percent of hatch.

Dose (Gy)	Hatching (%) ± SE	Mean number of eggs collected per flask (± SE)	Total number of eggs collected
0	80.60 ± 8.06	120.90 ± 39.50	3384
20	39.30 ± 22.18	37.90 ± 13.36	530
40	—	0	0
60	—	0	0

emergence, the metamorphosis is almost complete and the detrimental effects of irradiation on organs with low metabolic rate is minimized. However, the spermatogenesis is still ongoing, spermatogonia and spermatozoids are still differentiating and they constitute the main target for dominant lethal mutation induction.

Competitiveness of irradiated males is negatively correlated with the absorbed radiation dose (Calcagno 2001; Calcagno et al. 2002; Lux et al. 2002). Therefore, in order to optimize the efficiency of the SIT it is necessary to reach the best compromise between sterility and competitiveness (Parker & Mehta 2007). Taking into account the results obtained in Experiment 1 and the arguments discussed above, Experiment 2 was based on pupae irradiated 48 h before emergence.

The relationship between dose and percent egg hatch observed in the present research was similar to that observed in other tephritid species. The results are consistent with the "one-hit" hypothesis (La Chance & Graham 1984) which predicts a linear response at low doses, but as the dose increases an increasing proportion of sperm carry multiple dominant lethal mutations. One dominant lethal mutation, however, is sufficient to cause lethality. Furthermore, high irradiation doses produce unwanted side effects reducing the relative efficiency of the radiation (Hooper 1989).

According to the results presented here, relatively low doses of radiation, e.g., 20-40 Gy, cause 90-97% sterility. This agrees with other authors who observed that 40 Gy can sterilize *A. fraterculus* (González et al. 1971), 50 Gy (48 h before emergence) produces 100% male sterility in *A. suspensa* (Walder & Calkins 1993), 60 Gy (24-48 h before emergence) produces high levels of sterility in *A. obliqua* (Toledo 1993), and 40 Gy at 96 h before emergence causes 100% male sterility in *A ludens* (Rhode et al. 1961).

We observed that the induced sterility remained across the 7 egg collections made over a month for all treatments. This consistency among egg collections indicates that males do not recover their fertility during this period for any of the doses considered. These results are consistent with those of González et al. (1971) in the same species, and those of Velasco & Enkerlin (1982) in *A. ludens*.

In tephritid females the oviposition is reduced as the irradiation dose increases. Moreover, the eggs laid show evidence of dominant lethal mutations. The doses that cause males sterility also completely inhibit oviposition in females (Burditt et al. 1975; Calkins et al. 1988; Hooper 1989). According to Velasco & Enkerlin (1982) females of *A. ludens* are more susceptible to radiation than males are, showing effects with extremely low doses (5-20 Gy). The reason is that 48 h before emergence the ovaries are in an early developmental stage and the radiation, depending on the dose, may cause complete ovarian atrophy (Walder & Calkins 1992).

According to our results, the irradiation of females of *A. fraterculus* results in a reduction in the number of eggs laid, compared with non-irradiated females after mating to fertile males. Doses of 20 Gy induced a 40% reduction in egg hatch and a 67% reduction in egg laying. The dose of 40 Gy was sufficient to induce complete sterility by preventing egg laying. The fact that irradiated females are not able to lay eggs is favorable to SIT implementation based on bisexual laboratory strains because the potential damage to some fruits (stings) by released females would be eliminated.

The analysis of sperm transfer indicated that empty spermathecae can be found in females exposed to both non-irradiated and irradiated males. The difference in the proportions of empty spermatecae between control and males irradiated with 60 Gy was not significant. Although we did not quantify the number of sperm transferred, our results indicate that the sterility of irradiated males should be attributed to the induction of dominant lethal mutations and not to atrophy of testis, seminal ducts, or aspermia.

Taken as a whole the results obtained in the present work support the use of a dose of 70 Gy applied 48 h before adult emergence to induce 100% sterility in males and females of *A. fraterculus*.

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