Use of the Sterile Insect Technique against *Aedes albopictus* in Italy: First Results of a Pilot Trial

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ABSTRACT In Europe, the mosquito *Aedes albopictus* (Skuse) is widespread in Italy, Albania and most probably in neighbouring Montenegro. Recent introductions have also been reported in France, Spain and southern Switzerland. In Italy, the species is currently recognized as the most noxious mosquito, thus requiring the implementation of intensive control programmes. *Ae. albopictus* is also a potential vector of human diseases, which has raised the issue of whether eradication campaigns are called for. This species is particularly suitable for application of the sterile insect technique (SIT) because of its urban-related distribution, recent introduction, low active dispersal potential, low population density which may be maintained by conventional control measures, and ease of mass-rearing. In 1999, a programme was initiated that focused on the application of the SIT against *Ae. albopictus*. A pilot rearing facility, targeted at the production of up to 20 000 male pupae per week has been established. Blood feeding is performed with a thermostatically controlled device using defibrinated bovine blood, egg hatching is stimulated with a nutrient broth culture, and egg counts conducted automatically. Larval density and larval diet are still being investigated in order to improve productivity, the separation of males is currently conducted at the pupal stage using calibrated metal sieves, and irradiation studies are performed at the ^{60}Co plant Calliope, Italian National Agency for New Technologies, Energy and the Environment in Rome. During the summer of 2004, eight weekly sterile male pupal releases were organized in Rimini to evaluate sterile male performances in the field against a natural population. A significant difference was observed in the release area compared with the control area when the effects on egg fertility and egg density were cumulated. It is planned to continue the programme on a larger scale to improve rearing efficiency and obtain a preliminary benefit/cost evaluation.

KEY WORDS *Aedes albopictus*, Asian tiger mosquito, SIT, pilot release, Italy, rearing

1. Introduction

The Asian tiger mosquito *Aedes albopictus* (Skuse) has invaded several countries in recent years, mainly due to passive transportation in used tyres (Reiter and Sprenger 1987). In Europe, the species was first recorded in Albania in 1979 (Adhami and Murati 1987), in Italy in 1990 (Sabatini et al. 1990), in France in 1999 (Schaffner and Karch 1999), in Belgium in 2000 (Schaffner et al. 2004), in Montenegro in 2001 (Petric et al. 2003) and in Switzerland in 2003 (Flacio et al. 2004). Other countries have already been invaded or are about to be so in the Middle East, Africa and the Americas.

In Italy, establishment appears to have been rapid, mainly due to passive transporta-

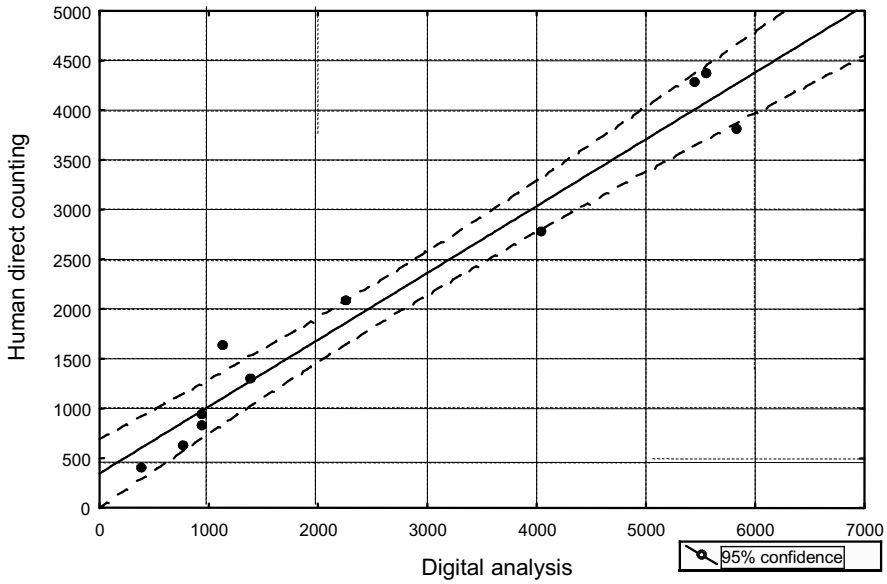

Figure 1. *The correlation between automatic egg counting versus human direct counting (Equation: Human direct counting = 345.5 + 0.67Digital analysis, r = 0.979).*

tion of adults inside vehicles, and the species is currently found in twelve regions (Urbanelli et al. 2000, Romi 2001).

In its area of origin (Asia), *Ae. albopictus* is known to be an important vector of many arboviruses including yellow fever and dengue. Moreover, it is also capable of transmitting indigenous arboviruses in newly invaded areas (Shroyer 1986), as well as filariasis (*Dirofilaria immitis* Leidy and *Dirofilaria repens* Railleiet and Henry), and other arboviruses like Sindbis, Chikungunya, West Nile and Rift Valley (Cancrini et al. 1992, Mitchell 1995). Finally, this species can also be a serious nuisance because of its high anthropophily and painful bite.

The species is mainly found in urban and peri-urban areas where it develops in man-

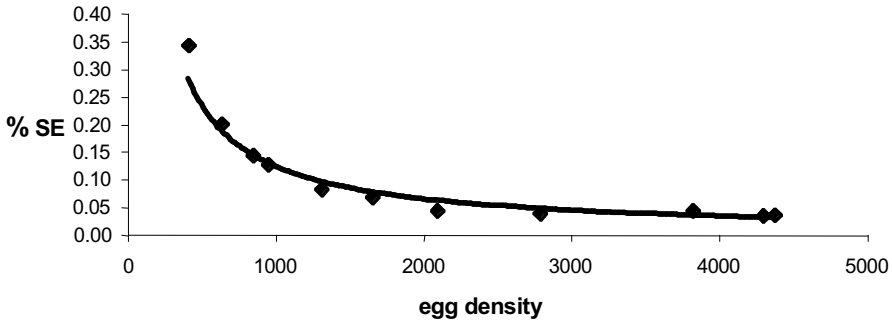

Figure 2. *Digital egg counting standard error versus human direct counting at different egg densities (Y = 66.49 x - 0.909 , R^2= 0.948).*

made water containers. This "island" distribution and the mosquito's low active dispersal capability make it a potential candidate for the application of the sterile insect technique (SIT) as a complement to other control tactics already being implemented.

In 1999, a project financed by local funds (see acknowledgements) was started to investigate the feasibility of applying the SIT against *Ae. albopictus* in Italy.

2. Mosquito Rearing

2.1. Adult Maintenance

The colony used for the experiments originated from field-collected eggs from Desenzano del Garda (in the Province of Brescia), in the north of Italy in 1993, and has been routinely mixed with wild specimens collected from other areas in northern Italy. Mosquitoes were kept in an insectary under standard laboratory conditions (27 ± 1°C, 85% relative humidity, 15 hour scotophase). Adults were kept permanently in plexiglass cages (50 x 50 x 60 centimetres) with a supply of 10% sucrose solution to which they had constant access. Females were also provided with fresh mechanically defibrinated bovine blood using a special temperature control apparatus (Bellini et al. 2002). Eggs were laid on filter paper placed inside black plastic containers containing water, removed daily from the adult cages, left to dry in the climate chamber for 24 hours and than placed in a closed plastic box with a saturated solution of K_2SO_4. Using this method, eggs could be kept alive for a few months. When needed, the filter papers with the eggs were put directly in water. Larvae were fed on crushed dry cat food (Friskies® Adults) and kept in plastic larval trays containing dechlorinated aerated water.

2.2. Egg Counting

In order to rear larvae at fixed densities a method for automatic and rapid egg counting must be available. Eggs are black and laid individually on a white paper substrate in variable densities. By using an open source image processing and analysis programme (ImageJ, United States National Institute of Health) it was possible to achieve satisfactory egg counting accuracy by scanning eggs on the filter paper. The correlation between the digital analysis data and conventional direct counting was satisfactory (Fig. 1), as was the standard error distribution of the egg density range in current use (2000-4000 eggs per

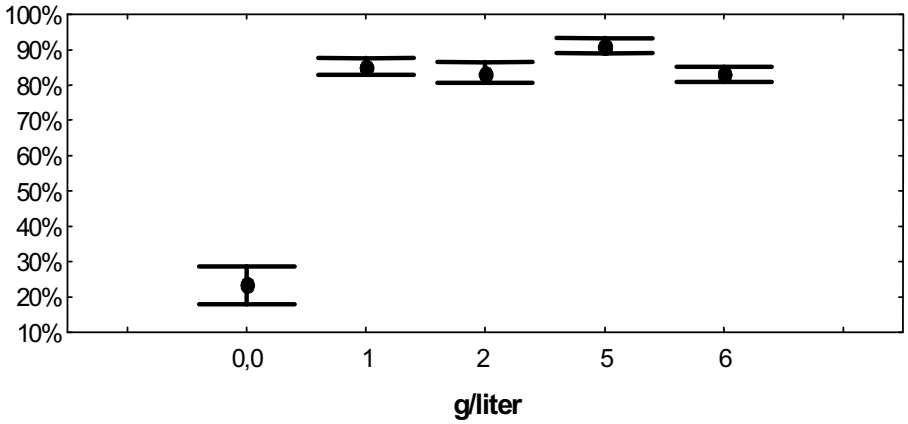

Figure 3. *Percentage of* Aedes albopictus *eggs hatched at different nutrient broth concentrations.*

paper) (Fig. 2).

2.3. Egg Hatching

Ae. albopictus eggs are generally difficult to hatch. Methods were therefore tested to standardize egg hatching in order to obtain a sufficiently precise and synchronized number of L_1 larvae from a known number of eggs. A nutrient broth culture was evaluated in order to deoxygenate the water (Barbosa and Peters 1969, Novak and Shroyer 1978).

Hermetically sealed glass jars (1 litre volume) with 700 millilitres of dechlorinated tap water containing 200-500 *Ae. albopictus* eggs were used to which nutrient broth at different concentrations was added. L_1 larval counts were made 24 hours after egg immersion. Five trials were carried out at the same temperature (27 ± 1°C). The results are illustrated in Fig. 3.

The broth had a strong stimulatory effect on egg hatching at all the concentrations tested with a consistently high percentage of hatching. As there appeared to be no significant difference between the effects obtained with different concentrations, 1 g/litre is currently used. Having observed that the broth had negative effects on larval development, young larvae were removed from the hatching solution. Further investigation is underway to find a way to avoid filtration of L_1 larvae by reducing the broth concentration or changing its composition.

2.4. Larval Density

Studies on larval density were conducted in rectangular white plastic trays (30 x 21 x 8 centimetres) containing 2.5 litres of dechlorinated water. Larvae were provided with standard larval food (Friskies® Adults dry cat food), throughout their development, at a fixed concentration of 4 milligrams per larva, of which 10% was given on day one, 45% on day two and 45% on day five. Evidence obtained from previous studies regarding pupal production (in relation to the initial number of L_1 larvae), and larval development time (calculated from the period between egg immersion and pupation), suggested that better results would be achieved with 1000 larvae per tray than with 100, 500, 750, 1250 and 1500 larvae per tray (Bellini et al. 2002). Following observations made by Teng and Apperson (2000) and Briegel (2003), larger trays (41 x 31 x 11 centimetres) containing three litres of water with a higher larval density and a new larval diet are being evaluated. Some recent results on the effect of larval density on pupal production using the conventional diet are summarized in Fig. 4.

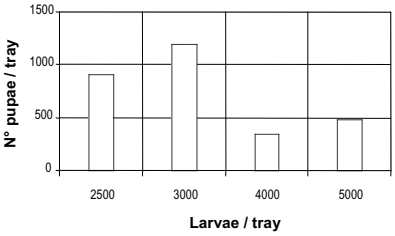

Figure 4. *The production of* Aedes albopictus *pupae at different larval densities.*

Attempts to improve the larval diet involved adding dried brewer's yeast to the standard diet. The dose was fixed at 2.5 milligrams per larva of Friskies® Adults + 1.5 milligrams per larva of yeast. As with the standard method, 10% was given on day one,

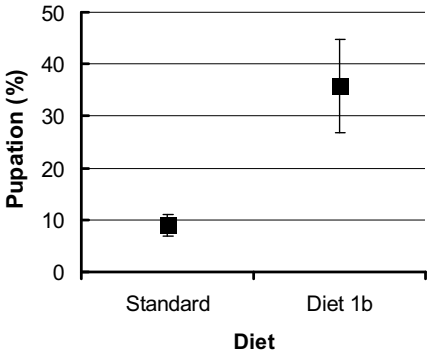

Figure 5. *Mean (± SE) percentage pupation using different larval diets at a density of 1500 larvae per litre (trays with three litres of water).*

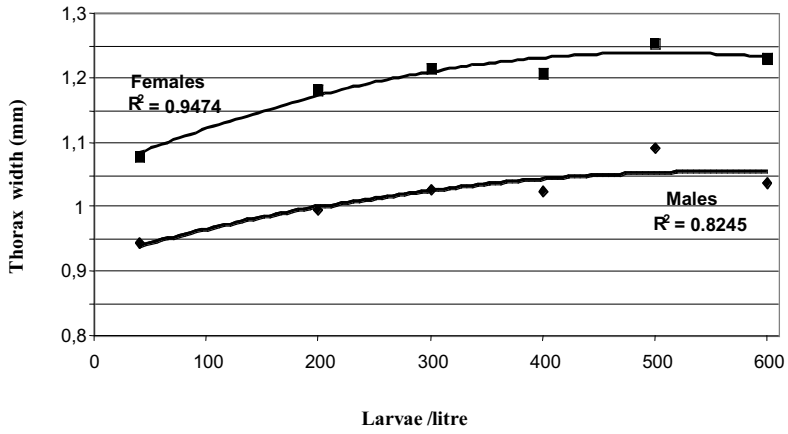

Figure 6. *Size of female and male pupae of* Aedes albopictus *in relation to different larval rearing densities.*

45% on day two and 45% on day five. Results are summarized in Fig. 5. A clear increase in pupal production was achieved when a density of 1500 larvae per litre was used.

2.5. Pupal Sexing

Considering previous findings on several mosquito species and personal observations regarding pupal size and sex in *Ae. albopictus* (Fig. 6), attention was focused on the sieving technique to achieve sex separation (McCray 1961, Sharma et al. 1972, Sharma et al. 1974). The metal sieves used (Giuliani®) have a round stainless steel frame (diameter 20 centimetres and height six centimetres), supporting a square mesh. Trials were conducted with 1250, 1400 and 1500 micron sieves tested independently or in succession. Currently, pupae are collected once per production cycle at a fixed time approximately 24 hours after the beginning of pupation to better exploit proterandry. Pupae are processed together with larvae by being placed in water at 35°C with the sieve for 4-5 minutes. To make it easier to separate residual larvae from male pupae, one part per million of *Bacillus thuringiensis israelensis* (de Barjac 1980) is added to the container to kill the larvae (but not the pupae, as they do not feed) used to transport pupae to the radiation source instead of using the ice technique (Ansari et al. 1975a).

Experiments are planned to check whether exposure to *B. t. israelensis* during the larval/pupal stage has any negative effect on adult males (Zahiri and Mulla 2005). Separated male pupae are collected for radiation while non-separated male and female pupae are reintroduced into the colony. Current average male pupal productivity is ca 15-25% (based on the initial number of L_1 larvae), with the presence of females ranging from 1-3%. Tests are presently being conducted with the aim of increasing male productivity (harvesting pupae at 36 hours instead of 24 hours from the beginning of pupation), and reducing the proportion of females in the released material (shortening the time of sieving, testing sieves with a rectangular mesh, etc.).

2.6. Pupal Irradiation

Irradiation is performed with a ^{60}Co source (Calliope) at the Italian National Agency for New Technologies, Energy and the Environment (ENEA), Rome by exposing pupae in water using a special plexiglass device. External dimensions are 41 x 60 x 3 centimetres (six stacked trays 41 x 10 x 3 cen-

Table 1. *Mean longevity of male* Aedes albopictus *exposed to increasing doses of radiation (100 individuals per cage, 27 ± 1°C, 85% relative humidity, 15 hours scotophase).*

Gy	Longevity of irradiated males (days)			
	No. of replicates	Mean	SD	Test Newman -Keuls
Control	4	35.3	2.3	a
60	3	22.2	1.8	ab
80	4	16.4	6.9	ab
100	4	13.1	3.6	b
110	1	16.2	---	ab

timetres each), allowing the radiation of 20 000 pupae per session. The height of the trays has now been reduced from ten to five centimetres in order to increase the number of pupae.

Dose response studies were conducted in the range of 60-110 Gy at dose rates of 186, 462, and 1190 Gy/hour. Male longevity, male sterility, possible male fertility recovery and sterile male versus fertile male competitiveness were assessed in 40 x 40 x 40 centimetre cages under laboratory conditions. Egg fertility was assessed by standard conditioning of the eggs and application of the hatching method as described above.

The current dose employed for the field pilot studies is 80 Gy, given at a dose rate of 186 Gy/hour, which results in complete male sterility and good competitiveness in cage studies. Further studies are in progress to evaluate the possibility of reducing the dose of radiation and to obtain greater insight into the effect of colonization on the field fitness of sterile males.

Male longevity was affected by radiation at all the doses tested, but not to such an extent as to preclude the planning of weekly releases (Table 1). Surprisingly, 110 Gy gave higher longevity than 100 Gy, and similar longevity to that obtained at 80 Gy.

Tests to establish the optimal radiation dose were conducted prior to the use of the nutrient broth technique, thus making the results difficult to interpret (see low egg hatching in control, Table 2). However, at

Table 2. *Fertility (% egg hatch) following irradiation of male* Aedes albopictus *pupae at different doses (462 Gy per hour if not specified). Competition cages contained 50 sterile males, 50 fertile males and 50 virgin females. Sterile males cages contained 50 sterile males and 50 virgin females.*

	Eggs hatch (%)					
	Gy	Number of replicates	Mean	SD	Test Newman-Keuls	Competitiveness index
Control	0	4	34	27	a	-
Competition cages	60	2	24	20	ab	0.70
	80	3	23	21	ab	0.74
	100	3	27	27	ab	0.63
	110	1	12	8	bc	1.41
Sterile male cages	60	3	2	3	c	-
	80	4	1	2	c	-
	100	4	0	1	c	-
	110	1	0	0	c	-
	60 (1190 Gy/h)	1	3	3	c	-
	80 (1190 Gy/h)	1	0	0	c	-

Table 3. *ANOVA on estimated number of F_1 progeny (i.e. no. eggs x egg hatch) in the sterile male release area as compared to the untreated control area.*

	Mean	SD	n	Diff.	SD	*t*	df	*P*
Release area	36.21	13.53						
Control area	56.77	26.84	9	-20.55	24.72	-2.49	8	0.037

present, 80 Gy is considered the minimum acceptable sterilizing dose although males showed some residual fertility. The relationship between radiation sensitivity and pupal age is currently being investigated (Table 2).

Competitiveness studies in cages showed that the performance of irradiated males was reduced compared with normal males of the same age (Table 2). No clear relationship was evident between radiation dose and competitiveness in the range of doses tested. Again surprisingly, the competitiveness of males irradiated with 110 Gy seemed better than those irradiated at lower doses. Further investigation is needed in order to clarify the relationships between (1) pupal age and sensitivity to radiation in the context of a large-scale mass rearing, and (2) sterile male competitiveness in wind tunnels and different radiation protocols.

Female longevity was also reduced by radiation (data not shown), but females were still able to take several blood meals with complete suppression of fecundity at the tested doses of 70-75 Gy. Therefore the release of females, while having an obvious negative effect on the efficiency of the SIT and therefore needs to be reduced to a minimum, is not an interfering factor in the assessment of field release efficacy.

3. Pilot Field Test 2004

During the summer of 2004, a pilot field test was conducted in the centre of Rimini, northern Italy, where the species is well established. Eight weekly releases were implemented during the period 16 June-4 August in an area of ten hectares, while a similar area was used as a control. Regular control activities (larvicide and source reduction) were conducted on the whole urban area by operators who were not informed about the experimental location. Pupae were placed in plastic jars on the ground at 40-45 fixed sites in shaded environments. It is estimated that 50 000 adults emerged from a total of 65 000 pupae. This corresponds to 100-1000 males per hectare per week. Egg monitoring was conducted using standard ovitraps (Bellini et al. 1996), from two weeks before the beginning of releases to two weeks after the releases ended. In each release and control area, 20 ovitraps were positioned at fixed sites and checked weekly. Field-collected eggs were processed regularly to check fertility levels.

Fig. 7 shows the data collected during the trial. A slight, but continuous reduction in the fertility of eggs collected in the release area was recorded, while egg fertility remained quite steady in the control area throughout the whole season. In the release area, egg fertility had increased to a level similar to that of the control area by two weeks after the end of the releases. The trend in the number of field-collected eggs show fluctuations during the season, tending to increase late in the summer in the control area, as is usual, while remaining quite stable at a lower level in the release area.

Comparing the average number of F_1 progeny (number of eggs x egg hatch) produced in the release and control areas over the whole season, a significant reduction was recorded in the release area (Table 3). Despite the low number of sterile males released and the small size of the release area with a presumable high immigration of mated females,

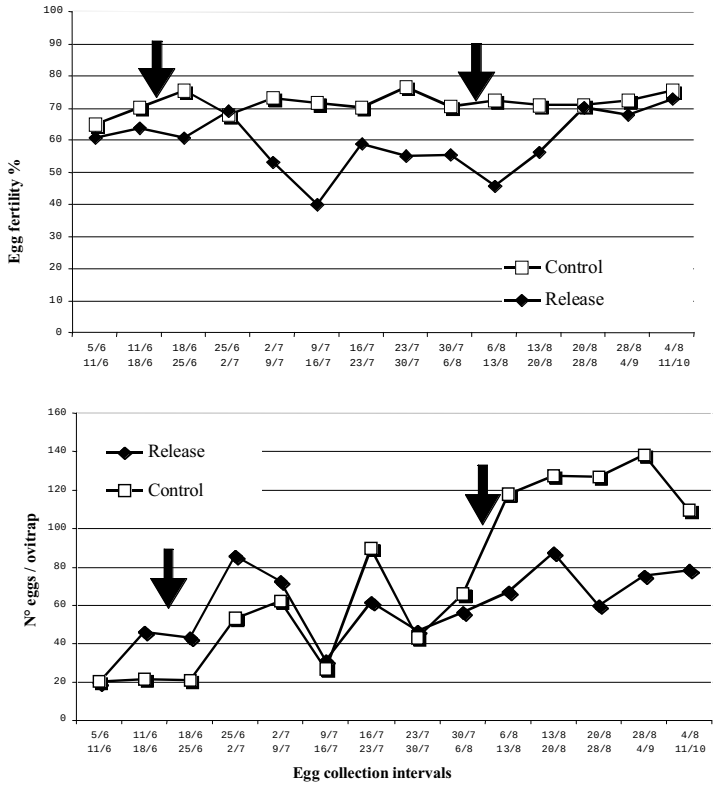

Figure 7. *Number of eggs (lower) and their fertility (upper) collected in sterile male-treated and untreated control areas (Rimini in 2004). Arrows show the beginning and the end of the releases.*

this positive result encourages continuation of the programme.

4. Considerations for the Future

Integrated application of the SIT for *Ae. albopictus* has not been attempted in the past, although studies of this nature have been made on several other mosquito species including *Aedes aegypti* (L.) which is a closely related species and may be taken as a useful example (McCray 1961, Weidhaas and Schmidt 1963, White 1966, Barbosa and Peters 1969, Jacob and Bevier 1969, Ansari et al. 1975b, Seawright et al. 1975, Curtis et al. 1976, Grover et al. 1976, Lorimer et al. 1976, McDonald et al. 1977, Ogah and Juma 1977, Hagen and Grunewald 1990).

Most of these studies were conducted in the 1970s although at a scale not large enough to be cost-effective and some programmes suffered from political problems. However, new technologies make the SIT approach more feasible and of high potential for future application.

Although positive, the preliminary results reported here need to be confirmed through a larger trial, conducted in a more isolated urban area. Field investigations should aim at evaluating: (1) the number of sterile males to be released, (2) the period covered by the releases, (3) the dispersal of sterile males and thus the spatial distribution of the release stations, as well as (4) both the costs and benefits

in comparison with conventional control methods.

With respect to mass-rearing techniques, further work is needed on the following aspects: (1) decreasing the nutrient broth concentration or changing the broth type in order to avoid negative effects on larval development and to eliminate L_1 filtration, (2) improving the rearing efficiency by increasing larval density through introducing new larval diets, (3) increasing pupal production by sieving at 30-36 hours after the start of pupation instead of at 24 hours, (4) improving sexing accuracy by testing new types of sieves or alternative methods (e.g. near-infrared spectroscopy), (5) improving sterile male competitiveness by reducing the radiation dose (which may be feasible by exploiting the pupal age-radiation sensitivity relationship), (6) developing larger cages or a greenhouse for colony maintenance, (7) irradiating adult males instead of pupae, and (8) screening new insect growth regulators for their sterilizing effect.

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