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Authors: Guerfali, Meriem M'saad, Parker, Andrew, Fadhl, Salma, Hemdane, Heitham, Raies, Aly, et al.

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FITNESS AND REPRODUCTIVE POTENTIAL OF IRRADIATED MASS-REARED MEDITERRANEAN FRUIT FLY MALES *CERATITIS CAPITATA* (DIPTERA: TEPHRITIDAE): LOWERING RADIATION DOSES

MERIEM M'SAAD GUERFALI[1], ANDREW PARKER[2], SALMA FADHL[1], HEITHAM HEMDANE[1], ALY RAIES[3] AND CLAUDE CHEVRIER[4]

[1]Unité de Recherche:utilisation médicale et agricoles des techniques nucléaires CNSTN UR01, Centre National des Sciences et Technologies Nucléaires, Technopole Sidi Thabet, 2020, Tunis, Tunisie

[2]FAO/IAEA Agriculture and Biotechnology Laboratory, A-2444Seibersdorf, Austria

[3]Laboratoire de Biomolécules Actives et de Microbiologie, Université Tunis El Manar Faculté des Sciences Naturelles de Tunis Campus Universitaire Tunis 2092 Tunisie

[4]Institut de recherche sur la Biologie de l'Insecte (IRBI) UMRCNRS 6035, UFR Sciences et Techniques 37200 Tours, France

ABSTRACT

Sterile insect technique programs continue to use high radiation doses to sterilize *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), without always considering the relationship between sterility and competitiveness. The aims of the present work were to verify, under laboratory conditions, the assumptions made by Parker and Mehta (2007) regarding the relationships between the level of residual fertility, competitiveness and sperm transfer ratio of mass-reared *C. capitata* males, and radiation dose. The males were irradiated in the pupal stage at doses ranging from 50 to 145 Gy. Our results show that radiation dose has a linear relationship with both competitiveness and fertility after suitable transformation. We could show that there is a clear optimum radiation dose at a dose dependent on the over-flooding ratio achieved.

Key Words: *Ceratitis capitata* (Diptera: Tephritidae), Sterility, Competitiveness, Radiation dose, SIT (Sterile Insect Technique), Optimization.

RESUMEN

Los programas que usan la técnica del insecto estéril continúan utilizando la dosis más alta de radiación para esterilizar *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) sin considerar la relación existente entre la esterilidad y la competitividad. Los objetivos del presente trabajo fueron verificar, en condiciones de laboratorio, los supuestos de Parker and Mehta (2007), sobre las relaciones entre el nivel de fertilidad residual, la competitividad y transferencia de esperma de los machos de *C. capitata* criados masivamente y las dosis de radiación. Los machos fueron irradiados en estado de pupa en un rango de dosis entre 50 a 145 Gy. Nuestros resultados muestran que la dosis de radicación tiene una relación linear con la competitividad y la fertilidad después de una transformación apropiada. Nosotros pudimos demostrar que existe una clara dosis de radiación óptima a una dosis dependiente en la tasa de la inundación alcanzada.

The Sterile Insect Technique (SIT) is a biological, environmentally friendly method of pest suppression or elimination that fits well into the area-wide approach of preventive insect pest management (Knipling 1955). This technique has become the main strategy for the area-wide suppression or elimination of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) populations. At present, ionizing radiation is the only method used to induce infertility in insects other than the boll weevil on a practical scale. Radiation induces dominant lethal mutations, which result in early embryonic death, but can also provoke damage to somatic cells resulting in reduced competitiveness of the males (Muller 1927; Muller 1950; Sakurai et al. 1994; Barry et al. 2003; Calkins & Parker 2005; Robinson 2005; Lance & McInnis 2005).The dose used to induce sterility is of prime importance in programs involving the release of sterile insects. Doses that are too low result in insects that are not sufficiently sterile, while doses that are too high will generate males that are poor competitors relative to wild males in achieving matings with wild females (Robinson et al. 2002). It is, therefore, crucial to optimize the balance between somatic and reproductive fitness and genetic sterility (Toledo et al. 2004).

Optimum effectiveness of the SIT requires that the mass-reared and irradiated males are well-distributed in the field and are competitive with wild males in terms of flight, mate-finding, mating behavior, and sperm transfer so an appropriate balance must be struck between reproductive sterility and somatic damage.

Males differ in their reproductive quality according to their age, mating experience, size, and genotype (Bangham et al. 2002; Reeve et al. 2002), or as a result of sterilization by chemicals or ionizing radiation (Hooper 1972; Lux et al. 2002a; Lux et al. 2002b) leading to potential differences in their competitive ability to access females (Hooper & Katiyar 1971; Lux et al. 2002b; Fawcett & Johnstone 2003a, 2003b). Sterile males perform less courtship and obtain significantly fewer first and second matings than fertile males as a consequence of the somatic damage.

Irradiated males must be capable of transferring the appropriate amount of sperm and accessory gland fluid during mating. In many species, male fitness is often limited by their competitive abilities, because the potential fertilizing capacity of the males exceeds their actual mating success. Generally, female fertilization is considered as a threshold trait (at a given time, females are either inseminated or not), which may be misleading if there is a variation in the quality or quantity of available sperm or accessory fluid (Reinhardt 2001; Chevrier & Bressac 2002).

In an earlier laboratory study of irradiated and control medflies from a laboratory strain Seo et al. (1990) found that the irradiated males had inferior inseminating ability. The number of sperm stored by the mates of sterilized males was much lower than that of wild females from the same region (Eberhard 1999; Taylor et al. 2001).

The relationship between residual fertility and dose is sigmoid in form (Hooper 1972), and the relationship is normally linearized by a log/Probit (or normal equivalent deviate, NED) transformation. Lacking adequate published data, Parker and Mehta (2007) in their model assumed that the NED against log (dose) regression line for residual fertility is parallel to the NED competitiveness against the log (dose) line. This critical balance between sterility and competitiveness has rarely been investigated or discussed in sufficient detail, and few data have been presented in the literature in a form allowing appropriate analysis of this balance (Bakri et al. 2005). Likewise, with respect to *C. capitata*, there are no available data on the relationship between sperm transfer and radiation dose.

The aim of the present work was to verify, under laboratory conditions, the assumptions made by Parker and Mehta (2007) regarding the relationships between radiation dose, the level of residual fertility, and competitiveness, in mass-reared *C. capitata* males irradiated in the late pupal stage with doses ranging from 50 to 145 Gy. We also determined the ratio of sperm transferred to the female to the total sperm compliment of the male (sperm transfer ratio). The new data presented here for these 3 parameters enable these relationships to be analyzed in order to determine the optimum dose for *C. capitata* SIT programs to achieve the best possible combination of these 3 critical parameters.

MATERIALS AND METHODS

General Methods

The *C. capitata* were obtained from a colony of the VIENNA 8 genetic sexing strain (GSS) produced in the Tunisian Mediterranean fruit fly rearing facility in the Centre National des Sciences et Technologies Nucléaires (Willhoeft et al. 1996; Franz et al. 1996). The mass-rearing procedures were those recommended for *C. capitata* (GSS) and described by M'saad Guerfali et al. (2007).

Male pupae 2 d before emergence were irradiated in a Cobalt-60 irradiator designed for foodstuff and sterilization of medical equipment. An irradiation device was installed, designed specifically for the irradiation of *C. capitata* pupae, consisting of 4 turntables that make it possible to rotate the canisters holding the pupae within the radiation field. The axis of rotation is vertical and parallel to the source pencils. For dose optimization, male pupae were treated at the following radiation doses: 50, 60, 70, 80, 90, 100, 110, 120 and 145 Gy. Bakri et al. (2005) report a maximum dose of 145 Gy for sterilizing Diptera although the *C. capitata* pupae shipped from Hawaii to California are treated with 150 Gy. Gafchromic dosimeters, calibrated against an alanine/ESR dosimetry system, were used for the dose distribution measurements (ISO/ASTM 2005a; 2005b).

All the experiments used virgin males (control and irradiated) and females five days after emergence, when they had reached sexual maturity. Males and females were separated immediately after emergence and were provided with a yeast hydrolyzate and sugar mixture (Tanaka et al. 1970) and water. To count spermatozoa in females after copulation and in virgin males, the entire reproductive tract was removed and placed in a drop of Beadle solution (128.3 mM NaCl, 4.7 mM KCl, 2.3 mM $CaCl_2$) on a microscope slide, gently broken with entomological pins, and the drop stirred to spread the spermatozoa evenly. The spermatozoa were counted under a fluorescence microscope after ethanol fixation and DAPI (4', 6-diamidino-2-phenylindole) staining (Bressac & Chevrier 1998) following the procedure of Chevrier and Bressac (2002).

Radiation Sterilization Test

The sterility test was performed in small cages with 25 irradiated males exposed to the different radiation doses placed with 25 non-irradiated females. Five replications were carried out for each dose. Eggs were collected daily until 1000 eggs per replication had been collected. For each dose and replication, eggs were placed on a moistened, circular, black filter paper inside a Petri dish (90 mm × 15 mm) in order to ensure the necessary moisture for the eggs during incubation at 25 °C. Egg hatch was recorded 4 d after seeding (FAO/IAEA/USDA 2003).

Male Development, Survival, and Flight Ability after Irradiation

After irradiation, the pupae were placed in flight ability cylinders (10 cm high, 100 pupae per cylinder) for emergence (FAO/IAEA/USDA 2003). Each tube was coated with talcum powder to prevent the flies escaping by walking up the cylinder. The cylinders were placed in Plexiglas cages 30 cm wide × 40 cm deep. Emerging flies were collected continuously. The test results were recorded 72 h after set up. After emergence of males the percentage of male fliers was evaluated. The proportion of flies with malformations (deformed wings) was also recorded. Ten replicates were performed on the same daily batch.

The survival percentage was the proportion of adults that survived for 48 h without food or water (survival under stress). Within 2 h of emergence samples of 50 irradiated males per dose without food and water were placed in Petri dishes (90 mm diam × 15 mm high) with a 15 mm opening covered with netting. The dishes were maintained in the dark at 25 ± 1 °C and 60-70% RH for 48 h. Dead and live flies were counted (FAO/IAEA/USDA 2003). Ten replicates were performed per treatment on the same daily batch.

Ratio of Transferred to Produced Sperm as a Function of Radiation Dose

We evaluated the effect of radiation dose on mean potential fertility of males, sperm storage, and sperm transfer to females by treated males. In the first series of experiments, males irradiated with doses from 50 to 145 Gy were dissected, and the sperm in their seminal vesicles were counted (see general methods). In the second series of experiments, in order to estimate the number of sperm transferred to females in a single mating from males treated with different radiation doses, we isolated in individual tubes copulating pairs from the competitiveness experiments, and retained for the sperm transfer measurement. Ten pairs were isolated for each treatment and after copulation the number of spermatozoa in the female's spermathecae (see general methods) from a male treated with a given irradiation dose were counted. Using average values, the ratio of sperm in females to sperm produced was calculated indirectly.

Competitiveness

The competitiveness test was performed in small cages (40cm × 50cm × 40cm) with 25 irradiated males, 25 control males, and 25 females. To distinguish between males, a lacquer color spot (Marabuwer GmbH & Co.) was put on the thorax of each control male 24 h before the competition. The treated males were not marked. This mark did not affect mating ability (Lacoume et al. 2007). The experiment was carried out under laboratory conditions (23 ± 1 °C and 60% RH). Flies were fed with sugar: yeast mixture (3:1) and supplied with water until the experiment. When copulation occurred, the pair was noted and isolated in an individual tube to allow sperm transfer to be completed. Ten replications were made for each dose from the same daily batch, and the proportion of copulations achieved by treated males was recorded as the measure of competitiveness.

Statistical Analyses

All data were checked for normality using a QQ plot. To normalize the data we carried out a Probit transformation on residual fertility and competitiveness values and an angular transformation of data for flight ability, malformations and survival. Data are presented as detransformed means ± one standard error (SE). Differences between groups were tested using multiway and one-way ANOVA tests (Statgraphics 5.0). Significant differences between treatments were determined by Tukey's honestly significant difference (HSD) test at the 5% experiment wise probability level. Linear regression analysis was performed on the relationship between transformed residual fertility and competitiveness and the log of radiation dose using R (R Development Core Team 2010).

RESULTS

Relationship between radiation dose and fertility of *C. capitata*

The means ± one standard error (SE) of the transformed residual fertility are presented in Fig. 1. A highly significant linear regression was obtained ($F = 74.49$; df = 1, 36; $P << 0.001$),which explains 67.8% of the variability in the regression of residual fertility on radiation dose.

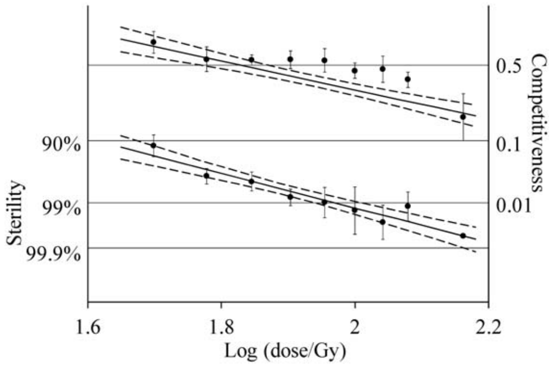

Fig. 1. Comparison of residual fertility (lower data) and competitiveness (upper data) of *C. capitata* treated at various radiation doses transformed to probit. Points are mean values with 95% CI. Linear regressions; residual fertility: y = 8.445 - 2.931x, df = 1, 36, F = -74.49, P <<< 0.001, R^2 = 0.674; competitiveness: y = 9.451 - 2.436x, df = 1, 88, F = 55.11, P<<< 0.001, R^2 = 0.385. Dashed lines are 95% CI of their respective regression lines. Light horizontal lines show the probit equivalent of various competitiveness and sterility levels.

Development, Survival and Flight Ability of Irradiated Males

Male Development. The proportion of untreated males emerging with malformation (deformed wings) was low (1.9 ± 0.6%). There was a significant correlation between radiation dose and the percentage of males emerging with malformations (R^2 = 0.11, P < 0.01), although none of the individual paired contrasts between untreated males (control) and treated males was significant (Fig. 2).

Survival Under Stress. Measurement of the survival of the irradiated males under stress for 48 h after their exposure to radiation showed that their longevity decreased significantly as the dose increased (Fig. 3) with survival significantly correlated to radiation dose (R^2 = 0.686, P << 0.001). Lower radiation doses (50, 60 Gy) did not have any significant effect on longevity (Tukey HSD 95%). Above 100 Gy, survival was less than the standard 50% recommended for mass-reared *C. capitata* GSS (FAO/IAEA/USDA 2003).

Flight Ability Of Irradiated Males. Flight ability was significantly affected by irradiation (R^2 = 0.434, P <<< 0.001) (Fig.4). Average flight ability for the controls was 88.4 ± 0.79%. No significant decrease was observed in males treated at 50 to 110 Gy, but there were significant decreases at 120 and 145 Gy (72.7 ± 3.06% and 66.6 ± 2.4% respectively).

Mating Potential of Sterilized Males

Ratio of Transferred to Produced Sperm. The fitness of irradiated males was evaluated in terms of competitiveness and ratio of the number of sperm transferred to the female to the total available sperm in the male (Fig. 5). There was no significant difference between the males treated at different radiation doses and the controls (F = 1.03; df = 9, 90; P = 0.4).

Competitiveness. In this series of experiments, competitiveness depended significantly on radiation dose (F = 12.2; df = 8, 90; P << 0.001) (Fig. 1). The competition between males treated with 50 Gy and control males for non-irradiated virgin females showed better mating success for the 50 Gy treated males than the controls (0.60, t = 2.273, df

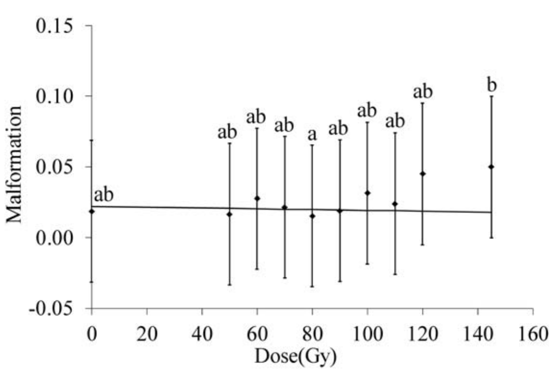

Fig. 2. Percentage malformation (detransformed means ± 95% CI) of *C. capitata* males that emerged from pupae treated with different radiation doses. The effect of radiation dose was analyzed by one-way ANOVA, linear regression of transformed percentage malformation; y = 0.0006x + 0.11, F = 2.74, df =9, 90, R^2 = 0.11, P = 0.0072. Points labeled with the same letter do not differ significantly at the 5% level (Tukey HSD test).

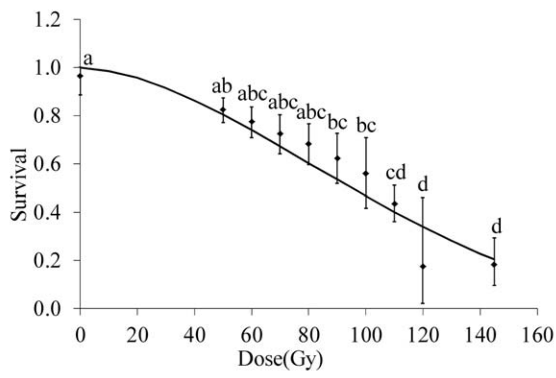

Fig. 3. Percentage survival (detransformed means ± 95% CI) of male *C. capitata* adults emerged from pupae treated with different radiation doses. The effect of radiation dose was analyzed by one-way ANOVA, linear regression of transformed percentage survival; y = 1.2361 -0.0054x, F = 104.6, df = 1, 48, R^2 = 0.686, P <<< 0.001. Points labeled with the same letter do not differ significantly at the 5% level (Tukey HSD test).

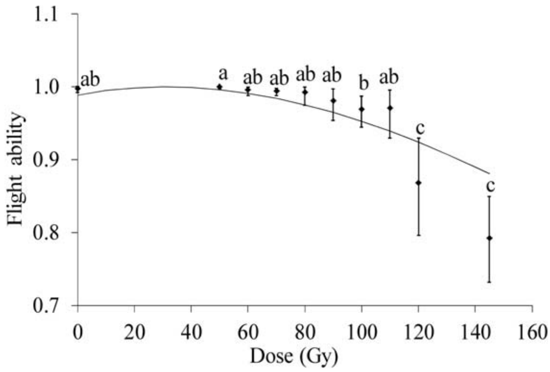

Fig. 4. Percentage flight ability (detransformed means ± 95% CI) of *C. capitata* males treated as pupae with different radiation doses. The effect of radiation dose was analyzed by one-way ANOVA, linear regression, y = 1.3122 - 0.00189x, F = 75.23, df = 1, 98, P <<<0.001, R^2 = 0.434. Points labeled with the same letter do not differ significantly at the 5% level (Tukey HSD test).

= 9, P = 0.04). This may be evidence of a hormetic effect, but the conditions of the experiment make this difficult to confirm. At the highest dose (145 Gy) male competitiveness showed a marked reduction compared to controls; irradiated males achieved only 15% of the matings.

Linear regression indicated a significant relationship between dose and competitiveness but explained only 38.5% of the variability in competitiveness.

Relationship between Residual Fertility and Competitiveness

Fig. 1 shows the relationships between fertility, competitiveness and the radiation dose with

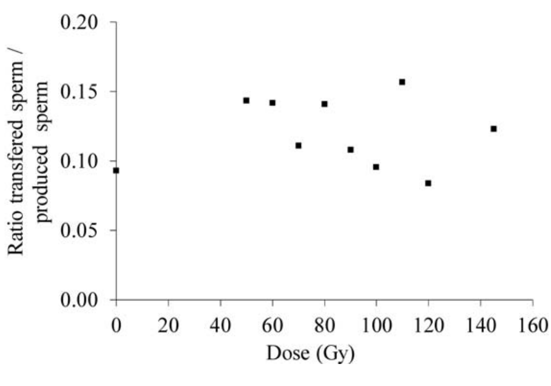

Fig. 5. Ratio of number of sperm transferred to virgin females to total number of sperm produced by *C. capitata* males treated as pupae with different radiation doses. The effect of radiation dose was analyzed by one-way ANOVA (F = 1, 03, df = 9, 90, P = 0.42).

linear regression lines and 95% confidence intervals for the regressions. The slope of these 2 regression lines did not differ significantly (t = 1.047; df = 2; P = 0.301).

Discussion

The SIT has become the principal tactic used in the area-wide strategy for controlling or eliminating *C. capitata*. However, past studies have confirmed a negative effect of irradiation on survival (Spates & Hightower 1970; Orozco et al. 1983; Ashley 1987; Mutika et al. 2002), flight ability (Calkins et al. 1996; Calkins & Parker 2005), mating competitiveness (Cayol et al. 2002) and sperm transfer (Mossinson & Yuval 2003). The radiation dose used to induce sterility is of prime importance in programs involving the release of sterile insects. Insects that receive too low a dose are not sufficiently sterile, while those receiving too high a dose may be uncompetitive, which may then require the release of a greater number of sterile insects (Robinson et al. 2002).

In our work, we used a range of doses from 50 to 145 Gy to determine whether a theoretical model that indicated that lower doses could be as effective for inducing sterility in a wild population as higher doses was supported. We confirmed that high doses give comparable sterility to that in other works (Hooper & Katiyar 1971; Hooper 1972) but our findings suggest that these high doses could also damage certain physiological processes leading to reduced survival, flight ability, and development malformations (Spates & Hightower 1970; Crystal & Whitten 1976). The major conclusion of our study is that lower doses will produce a greater reduction in a target population when overflooding ratios are low than the higher doses normally applied. Thus we believe that many programs are misguided in that they continue to be based on 99.9% sterility regardless of the overflooding ratio instead of adapting the sterility level to the ratio. In *Bactrocera tryoni* Froggatt, adequate sterility and improved flight quality has been achieved by reducing the target sterilizing dose (Collins et al. 2010).

On the other hand, we found no significant effect of radiation dose on sperm transfer. This result conflicts with previous studies on *C. capitata* that found that sterile males transferred fewer sperm than wild males, and that females that mated with irradiated males were more likely to re-mate than females mated with non-irradiated males (Seo et al. 1990; Bloem et al. 1993; Favet et al. 1995; Taylor et al. 2001; Mossinson & Yuval 2003). We postulate that there is an indirect effect of irradiation on reproductive potential by acting on the accessory glands perhaps decreasing fluid quantity, rather than on the number of spermatozoa.

The degree of competitiveness of irradiated males is of crucial importance in the SIT. Our results confirm the earlier work of Hooper (1970), who found that competitiveness decreases with increasing doses. Lower doses yield better results in terms of the combination of competitiveness and sterility (Parker & Mehta 2007). However, our competitiveness data were based on small cage studies, and it is known that confined spaces affect mating performance to the advantage of mass-reared insects, and mask the deleterious effects of irradiation and handling. This experiment needs to be repeated at least in a field cage in order to produce results relevant to operational programs (Economopoulos 1996).

Parker and Mehta (2007) developed a quantitative model for determining the optimum radiation dose based on fertility and competitiveness data. Lacking good published data sets, they had to make certain assumptions about the shape of the relationship between radiation dose and competitiveness, and between this relationship and the relationship between residual fertility and dose. The model would allow a program manager to estimate the correct radiation dose needed to maximize the impact of released irradiated males given the residual fertility, competitiveness, and achievable sterile to wild male ratios.

The model was based on the following assumptions:

1. The relationship between residual fertility and dose is linear after log/Probit transformation.
2. The relationship between competitiveness (Fried index, *C*) and dose is linear after log/Probit transformation.
3. These two linear relationships (after transformation) are parallel, with competitiveness higher than residual fertility.

The first of these assumptions is well-documented, but no suitable dataset was available to demonstrate the form of the relationship between competitiveness and dose. The aim of our work was to verify the assumptions made in that model by producing a complete data set under laboratory conditions.

Our results show that the competitiveness relationship is linear (after transformation) and highly significant (Fig. 1), although competitiveness was expressed as the proportion of males achieving mating (*p*) rather than the Fried index, where *C* is proportional to *p*/(1-*p*). The third assumption is also supported by our data, as the regression coefficients do not differ significantly.

Even if we had failed to support the third assumption, however, it would not invalidate the basic model. Reworking the model to account for the non-parallelism would simply move the optimum dose slightly higher if the competitiveness gradient is less (in absolute magnitude) than that of residual fertility, causing competitiveness to fall more slowly than fertility with increasing doses.

Using the regression relationships shown here, it is possible to calculate the impact on the number of fertile eggs that will be produced by releasing males treated at various doses and at different over-flooding ratios in the same way as Parker and Mehta's study (2007). The result is shown in Fig. 6 A for selected ratios varying between 1:1 and 1:1000 and for doses varying between 2 and 150 Gy. It can be seen that there is a clear optimum radiation dose, as predicted by Parker and Mehta (2007), and that this optimum occurs at a dose dependent on the over-flooding ratio achieved. For very low ratios, it occurs at about 50 Gy, rising to 80 Gy for intermediate ratios, and finally to 140 Gy only at the highest ratio. These low doses run counter to the current practice of using a dose that will produce sterility close to 100%. Fig. 6A implies that such a practice, at least for a suppression program or the early stages of an elimination program, is counterproductive and that a greater impact could be achieved with lower doses. The relationship be-

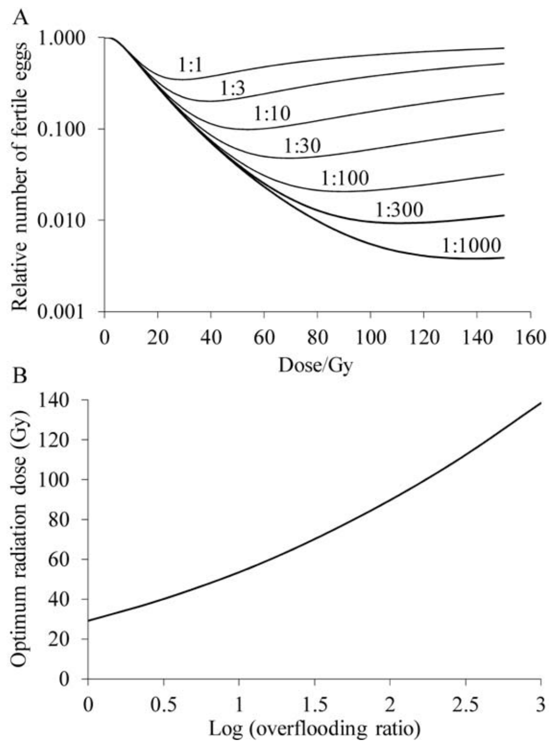

Fig. 6. A. Calculated values for the relative number of fertile eggs produced by releasing males treated at various doses and released at various over-flooding ratios. B. Calculated value for the radiation dose that will lead to the largest reduction in a population as a function of the overflooding ratio. The relationship is approximately of the form Optimum dose = 6 L^2 + 18 L + 30, where L = log (overflooding ratio).

tween the achievable overflooding ratio and the optimum radiation dose (the dose that will produce the maximum reduction in fertile eggs) is shown in Fig. 6B, and gives an impression of how the dose should be adjusted as a program progresses.

There are several factors that should be taken into account in interpreting these results:

1. The model extends to doses extrapolated outside the actual doses used.
2. The sterility measured for a given dose was high due to the inherent sterility of the VIENNA 8 *tsl* genetic sexing strain. However, in these experiments the fertility of the controls was lower than expected and may have distorted the fertility/dose relationship.
3. The experiments were conducted under laboratory conditions in small cages, conditions that are known to influence competitiveness (Economopoulos 1996). We do not, therefore, claim to have determined the optimum dose for a particular situation, but rather to have demonstrated the principle that a clear optimum exists at a lower dose than normally used.

The outcome in the field will also be influenced by the other factors measured here, survival, sperm transfer, and flight ability. Translating laboratory measures to field effects is not possible without adequate field validation, so we have not been able to quantify these effects and incorporate them in the model. However, it is clear from these results that increasing dose causes drops in both survival and flight ability, and therefore will make released sterile males less effective, resulting in the curves on the right-hand side of Fig. 6A rising faster from the optimum and concomitantly move the optimum to lower doses. The finding that sperm transfer was not affected by irradiation is counter to previous reports and would need further investigation to clarify what is happening.

For *C. capitata* suppression programs, the aim is to reduce the population to below a certain economic level. The aim should not be to achieve near 100% sterility but to find a balance between sterility and competitiveness which has rarely been investigated or discussed in sufficient detail, and few data have been presented in the literature in a form enabling adequate analysis (Bakri et al. 2005).

The cost and effectiveness of a control program using the SIT depend on the balance between sterility and competitiveness, but it appears that many operational programs with an SIT component do not achieve an appropriate balance. To conclude, our study demonstrates that it is often possible to reduce the dose used to sterilize *C. capitata*.

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