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Gamma-Ray Induced Dominant Lethal Mutations in Male Germ Cells of the Filariasis Mosquito Culex Pipiens Fatigans, Wiedemann, (Culex Quinquefasciatus. Say)

by

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Abstract

A study of the effects of gamma irradiation on different male germ cells of *Culex pipiens fatigans* was made.

Batches of 2-day old males were irradiated with gamma-rays with doses ranging from 500R to 10,000R and the treated males were individually mated to unirradiated virgin females (one treated male to three females for 3 days with a rest of 2 days for the males). Three such 3-day matings were carried out with fresh batches of virgin females and three successive broods were obtained from each male separately. The egg rafts obtained in the successive broods were screened separately for dominant lethality. The percentage number of unhatched eggs in each brood from females that showed spermatozoa in their spermathecae was used as the measure of dominant lethality.

The dominant lethal frequency increased linearly with dose in all the three broods up to a dose of 8000R. In the third brood the gradient was found to be half of that for the first-two broods. This indicates that a 50% recovery from radiation damage of the germ cells had taken place.

It has been found in *Drosophila* that the most radiosensitive stage in spermatogenesis is the late meiotic stage, and the least is the spermatogonial stage.

Our results for *Culex pipiens fatigans* agree with that for *Drosophila* in that the first two broods are most radiosensitive whilst the third brood is half as sensitive. The fact that sensitivity is least in the third brood would suggest that this brood has resulted from irradiated spermatogonia, as in the case of *Drosophila*. The first two broods seem to arise from a mixture of the later germ cell stages.

INTRODUCTION

Certain characteristics of radiation damage to various stages of male germ cells provide a clue to the identification of these stages of spermatogenesis. This aspect of radiation mutagenesis has been extensively studied in *Drosophila melanogaster* by Auerbach [1], Luning [2], Puro [3] and others. Similar studies have been carried out on other organisms, namely, in *Drosophila virilis* by Alexander [4] and in Mice by Russel [5]. Very similar conclusions have been arrived at by all these authors with regard to germ cell stages and the damage caused them by irradiations.

The damage to germ cells by irradiation can be studied by outcrossing irradiated young males over successive periods to unirradiated virgin females. When an adult male is exposed to irradiations all the existing germ cell stages in the testis are simultaneously irradiated. Spermatogenesis consists of a developing series of germ cells which may be in the following order, namely, spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa. The progenies obtained in successive matings, that is, successive broods, arise from successively younger germ cells.

These progenies can be tested for genetic effects such as dominant lethal mutations, recessive lethal mutations, translocations and recombinations. The frequencies of these mutations vary in the different broods and therefore could be used to determine and compare the different stages of spermatogenesis.

The present paper deals with brood analyses carried out after irradiation with gamma-rays on males of *Culex pipiens fatigans* which is the mosquito vector of filariasis, where only the induced dominant lethal mutation frequencies have been studied; the percentages of unhatched eggs were used as the measure of the dominant lethal frequencies. Caution has been taken to consider only egg rafts from females that showed spermatozoa in their spermathecae.

As mosquitoes are increasingly being studied for control by the "sterile insect release method" (SIRM), brood analyses of the above nature would be extremely useful to evaluate the chance of recovery from induced sterility and damage and for understanding the dynamics of spermatogenesis in mosquitoes. Except for the experiments of Grover and Pillai [8] with chemosterilants no definitive studies of brood analyses for genetic damage in mosquitoes have been carried out.

MATERIAL AND METHODS

Males of a colony of *Culex pipiens fatigans* maintained at this laboratory for over two years by continuous inbreeding were irradiated with gamma-rays. It is assumed that the genetic variability of these males was very low.

The irradiations were carried out with a Co^{60} gamma source (El Dorado Teletherapy Unit) at the Cancer Institute, Maharagama, Sri Lanka. The *Culex pipiens fatigans* males were placed in small polythene tubes (1.5x6cm) covered with a piece of muslin cloth at the open end. The tubes were exposed to gamma rays at a surface source distance of 50cm with a field of 25x20cm. The dose rate varied approximately from 210R per minute to 230R per minute. The total treatment doses given to various batches of male mosquitoes ranged from 500R to 10,000R. These irradiations were given to 2-day old males which had been fed on 10% sucrose solutions.

Altogether six experiments were carried out. The first two experiments when considered together were carried out with 500R, 1000R, 1500R, 2000R, 2500R, 3000R, 4000R, 5000R, 8000R and 10,000R treatment doses. For each experiment an untreated control was kept.

After considering the results obtained from these two experiments the two doses 3000R and 5000R were chosen to study brood patterns extensively in the four subsequent experiments.

The post irradiation procedures were the same for all experiments and was as follows: after a 2-day rest each male was placed with three unirradiated virgin females for three days for mating. The males were then separated and rested for two days before starting the second 3-day mating with three fresh virgin females. In this fashion three, 3-day matings were carried out with fresh batches of virgin females in order to obtain three successive broods.

After each mating the females were starved for one day and given chick blood meal. The blood meal was given by placing a chick overnight inside each mosquito cage. Each harem of engorged females was kept separately for two days till the blood meal was completely digested and then they were transferred to plastic cups, half filled with tap water, for egg laying. All the females (that is, those that had laid eggs, those that had died without laying and those that had refused the blood meal) were dissected and their spermathecae were examined for the presence of sperms and only those egg rafts of females that showed sperms in them were considered for estimating dominant lethal frequencies.

The egg rafts that were laid were transferred to small vials in which they hatched in about 24 hours. A day after the eggs hatched the remains of the egg rafts were taken on a piece of blotting paper and were observed under the microscope in a drop of xylene which separated the individual egg cases. Those eggs that were unhatched but were embryonated were also easily identified by observing the presence of eye spots in their embryos. The following data were recorded for each irradiated male for the three broods.

1. Number of eggs laid by each female mated to a single male.
2. Number of eggs found unhatched for each female.
3. Number of eggs found unhatched but embryonated for each female.
4. Number of females inseminated by each male during the three mating periods.

Some offspring from each raft of two experiments which were suspected of being semisterile were outbred for at least one more generation in order to detect semisterile mosquitoes. This was carried out not so much to measure

radiation damage but more so to detect translocations. To obtain such semi-sterile lines a few F_1 male offspring of each brood resulting from 3000R and 5000R treated males and which showed about 50% dominant lethal mutations were outcrossed to virgin females. If the semisterility bred true these lines were maintained over the generations by mating to fresh virgin females.

RESULTS

The percentages of unhatched eggs of each brood for each treated male were considered as the dominant lethal mutation frequencies. The numbers of inseminated females per treated male were used to estimate the mating ability of each male.

The pooled results obtained for the various doses for all six experiments for the three broods are given in Table 1. According to these results it can be seen that the induction of dominant lethal mutations increases with the irradiation dose, in all the three broods.

The results were statistically analysed using the least squares estimation method.

$$Y = a + bX,\ b = \Sigma xy/\Sigma x^2,\ r = \Sigma xy/\sqrt{\Sigma x^2 \Sigma y^2}$$

Y = dominant lethal mutation frequency, X = gamma dose

a = control value, x and y are deviations from their means.

b = regression coefficient and r = correlation coefficient

For the analyses only the values for doses O, 1000R, 3000R, 5000R and 8000R were considered. These were the doses common to at least two experiments.

Before calculating the regression coefficients, a correction was made for all the data using the Abbot's formula where the dominant lethal frequencies are experssed as percentages of the control value (relative dominant lethal frequencies). No data was obtained for the third brood of the 8000R treatment (this was because the males irradiated at high doses as 8000R, rarely mated in the third mating period). Therefore, only the doses 1000R, 3000R, 5000R and the control have been considered for correlation and regression analyses for the third brood.

Figure 1 shows the lines of best fit for the three broods and includes the respective regression and correlation coefficients. The correlation coefficients obtained for the three broods were 0.9996, 0.9309 and 0.9846 respectively. Since these values are close to unity it can be strongly asserted that the dominant lethal frequencies and doses are linearly correlated for all three broods.

Mating Period (Days)	4 to 7 (Brood 1)		9 to 12 (Brood 2)		14 to 17 (Brood 3)	
DOSE ROENTGENS	% Dominant Lethals	% Females Inseminated	% Dominant Lethals	% Females Inseminated	% Dominant Lethals	% Females Inseminated
0	5.0	72.1	8.0	75.7	7.0	53.6
500	12.8	76.0	25.9	60.0		
1000	16.0	78.0	13.0	67.0	12.0	50.0
1500	22.4	90.0	22.7	79.0		
2000	34.5	76.0	35.2	68.5	9.0	38.0
2500	38.3	71.0	55.3	60.0		
3000	38.9	77.8	37.1	52.7	16.2	32.5
4000	60.2	73.5	59.3	75.0		
5000	57.4	71.5	58.1	63.2	25.9	28.6
8000	90.4	76.0	94.2	55.0		
10000	97.6	64.0	96.8	31.0	93.5	

TABLE I - Combined Results of the Effect of Irradiation on Induced Percentage Dominant Lethal and Percentage Insemination in 3 Broods of *Culex pipiens fatigans*

Treatment (Dose In Roentgens)	*Brood*	*Number of Females Tested*	*Percentage Females inseminated*	*Total Number of Eggs Laid*	*Percentage Dominant Lethal*
Control	1	197	72.1	21,738	5.0
	2	194	75.7	24.414	8.0
	3	168	53.6	12,731	7.0
3000	1	234	77.8	26,639	38.9
	2	216	52.7	19,178	37.2
	3	215	32.5	13,135	16.2
5000	1	235	71.5	25,397	57.4
	2	231	63.2	22,750	58.1
	3	227	28.6	10,299	25.9

TABLE II – Results of the Effect of Irradiation (3000R and 5000R) on Percentage Dominant Lethal and Percentage Insemination in *Culex pipiens fatigans*

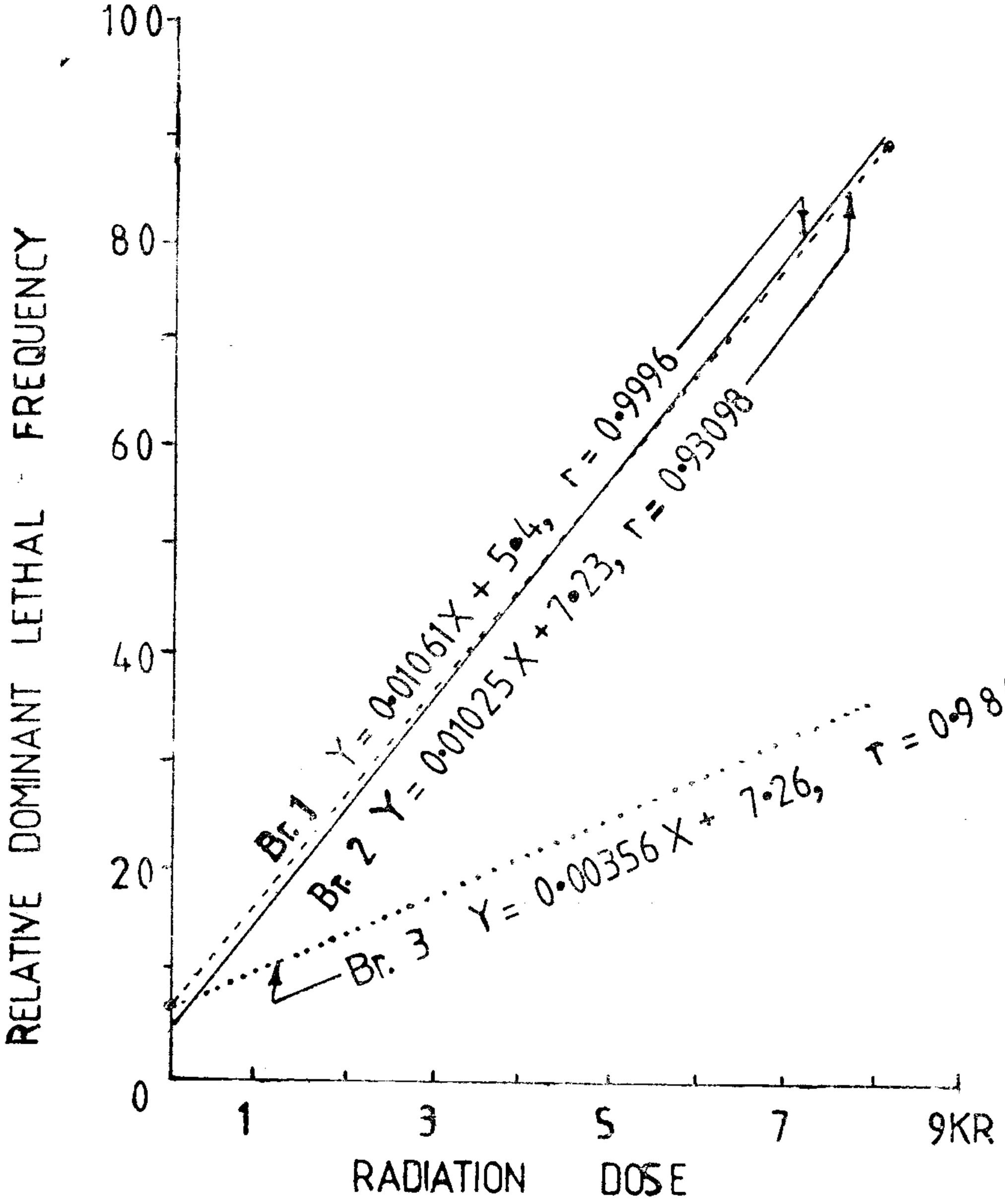

FIG 1 :— The dose response curves for the relative dominant lethal frequencies (r d.l.f.) for three broods of treated males of Culex pipiens fatigans.

(r. d. l. f. is the dominant lethal frequency expressed as the percentage of the control.)

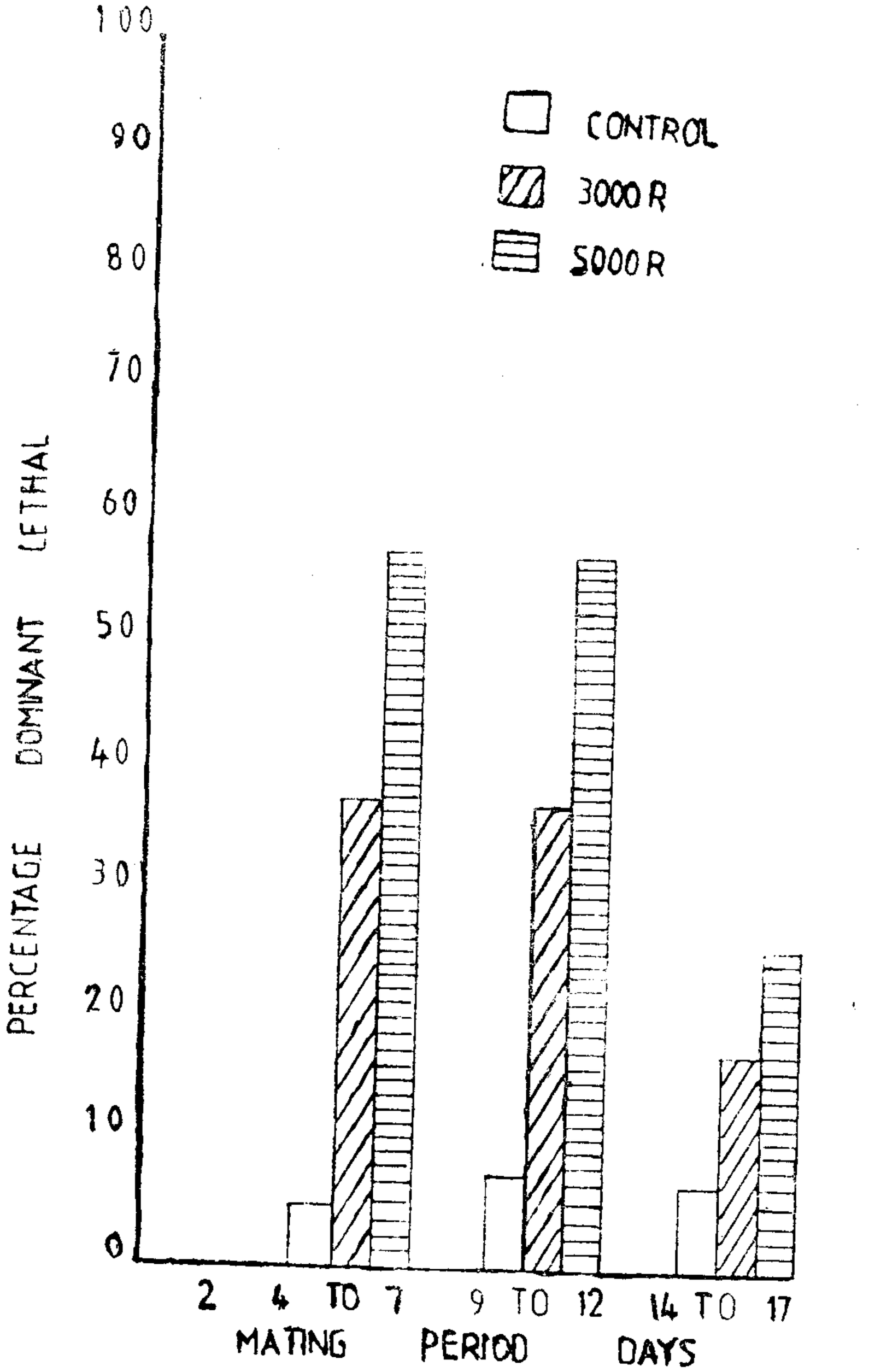

FIG 2 – Percentage of dominant lethals in the first three broods of CULEX PIPIENS FATIGANS for CONTROL, 3000 R AND 5000 R.

When the regression coefficients are compared for the three broods 1,2 and 3 it is seen that $b_1 = 0.1061$, $b_2 = 0.1025$ and $b_3 = 0.00356$. b_1 and b_2 being almost the same suggests that the rates of induction of dominant lethal mutations with dose are similar. However, the value $b_3 = 0.0036$ for the third brood being half of that for broods 1 and 2 shows that the rate is drastically reduced (50 %) implying a recovery from dominant lethal mutation frequencies in the third brood.

The pooled results of the six temporally replicated experiments which are given in Table 1 are set out in further detail in Table II.

From these results it can be seen that the induced dominant lethal frequency with 5000R is around 58 % in the first two broods ($br_1 = 57.4$ %, $br_2 = 58.1$ %) while it is only 26 % in the third brood. The values for dominant lethal frequencies with 3000R too show a similar variation where the first two broods show about 38 % ($br_1 = 38.9$ %, $br_2 = 37.2$ %) dominant lethal frequency which again shows a drastic drop to 16 % in the third brood. 50 % of the damaged cells for treatments between 3000R to 5000R are thus seen to be restituted in the third brood.

The similar dose frequencies for the first two broods and the conspicuous 50 % drop in the dominant lethal frequency in the third brood is clearly brought out in Figure 2.

Tables I and II also present the results of the percentages of females inseminated by the irradiated treated males in the three matings. Figure 3 clearly shows the difference of percentage insemination for each brood for the 3000R and 5000R treatments and the control.

The mating abilities of treated males as measured by the percentages of females inseminated by them show a clear decline for the third brood of the treated males when compared with the controls. For the doses of 3000R and 5000R the percentage insemination drops to 32.5 % and 28.6 % respectively in the third brood from, 77.8 % and 71.5 % respectively in the first brood. It should be noted however that there is a similar but lesser drop from 72.1 % in the first brood to 53.6 % in the third brood of the control itself.

It could be mentioned also that 29 separate semisterile lines of the above experiments. have been isolated by successive outcrossings of many F_1 males suspected for semisterility The inherited semi-sterility in these lines probably were due to chromosomal abberrations such as translocations or pericentric inversions. None of these lines was detected from brood 3 of any experiment. In 15 of the 29 lines that were isolated the semisterile character is closely linked to the male determining gene as only the males showed semi-sterility.

DISCUSSION

The phenomenon of radiation damage to various germ cell stages has been studied thoroughly in *Drosophila melanogaster* males. Auerbach [1] has studied the X-ray induced frequencies of autosomal recessive lethal mutations, recessive sex linked mutations, cross overs (recombinants), translocations and also the effect on fertility of males in *Drosophila melanogaster*. Luning as quoted by Auerbach [1] has studied in particular the dominant lethal frequencies induced by X-rays in the same species. Puro [3] reports more detailed work including visible mutants and clusters of third chromosome cross overs in *Drosophila melanogaster*. Other authors such as Sobels [6], Khishin [7] and Oster [8] have also studied different aspects of the same phenomenon in *Drosophila melanogaster*.

The studies of all the above workers, in particular the recessive lethal mutation and cross over studies, have led to the evaluation of the following sensitivity pattern of the *Drosophila melanogaster* testis to the mutagenic action of radiation. The pattern in the order of decreasing sensitivity is; spermatids and late spermatocytes > early spermatocytes > spermatozoa > spermatogonia.

The evaluation of the above sensitivity pattern was arrived at after considering many factors in the experiments of the above authors. These factors include consideration of different mating protocols, irradiation of males at different ages, scoring of various mutant types, etc. The mating protocol used in Auerbach's [1] experiments was one male to three virgin females for three days to obtain four broods without a resting period for the male. The age of males at the time of irradiation was 0 - 4 days. In Puro's experiments twenty broods were obtained by mating one male to two virgin females. The last seventeen of these broods were one day matings while the first three were 3-day matings.

The types of mutations scored in the various experiments are also important in evaluating the sensitivity pattern of germ cells because of their different mutation specificities. For example, excessive sterility is usually shown in cells treated at the late spermatogonial stage while the presence of clusters of mutations or cross overs indicates that they have arisen from a single stem cell which by a-sexual multiplication have given rise to clusters of similarly affected spermatozoa. Furthermore, absence of cross overs in a brood is evidence for cells treated after meiosis. Strictly genetical tests, like cross overs are the safest to use in determining the exact stage of the germ cells that have given rise to the various broods. Auerbach [1] has used both cross overs and recessive lethal mutations to determine this and her conclusions are most acceptable.

The use of percentage non-hatchabilities for estimating dominant lethality is fraught with some ambiguity as non-hatchability may be due to the presence of genuine dominant lethal mutations as well as due to cell injury, aspermia or lack of mating. Auerbach [1] and Alexander [4] have critically summarized these problems arising from estimation of dominant lethal frequencies.

In our experiments with mosquitoes we have estimated only dominant lethal frequencies from the non-hatchibility of eggs for three, 3-day broods and it would appear that no valid conclusion could be drawn from them. However, we have taken the precaution to count hatchabilities from egg rafts laid only by females which showed sperms in their spermathecae, thereby minimising the arbitrariness of the estimation of dominant lethal frequency. Therefore, by comparing our results with those of Auerbach's for *Drosophila melanogaster* we could gain some insight into the sensitivity of the mosquito testis, particularly because a clear-cut differential effect has been observed. According to Auerbach [1] broods 1 and 2 showed moderate sex linked lethal frequencies which are said to have arisen from irradiated spermatozoa and irradiated spermatocytes respectively. The second brood (brood 2) which showed the highest mutation frequency is said to have arisen from cells treated at spermiogenesis. The least sensitive were spermatogonia (brood 4) which showed the lowest mutation frequency.

In our experiments high dominant lethal frequencies were obtained in the first two broods and could have resulted from cells treated during and after meiosis. The low dominant lethal frequency in the third brood could have arisen from cells treated at the spermatogonial stage (stem cells). The absence of a peak mutation frequency corresponding to late spermatocytic and spermatid stages as shown in brood 2 of the *Drosophila* experiments is not reflected in our experiments. A probable reason for not obtaining a clear separation may perhaps lie in our mating protocol, that is, one male mated to three females for 3 days followed by a *rest of 2 days for the males.* This 2-day rest period would have interfered with the clear separation of germ cells into the different broods. Accumulation of developing spermatozoa could have given rise to a mixture of spermatozoa arising from irradiated spermatid and spermatocytic stages.

In any case, brood three clearly shows a depression of dominant lethal mutations which would imply that the relatively refractory spermatogonial stages are being sampled. The main reason put forward to explain this relative lack of sensitivity to irradiations by spermatogonial cells is germinal selection. We could also include as an explanation the restitution and repair of damage by the high metabolising state of these mitotically dividing cells.

Decreased mating ability of males in the third brood should actually increase non-hatchability of eggs and which has actually been observed in our experiments. But as we have left out those females which do not show sperms in their spermathecae, we have thereby eliminated sterility due to lack of mating ability in males or aspermia from estimations of dominant lethal frequencies. However, a very striking drop of mating ability in the third brood was recorded (see Results). It is seen from the Figure 3 that this decrease in mating

ability in the third brood is also shown by the control males and is perhaps due to the aging of males. A relatively higher drop in the irradiated males may be due to the treatment.

However, the fact that there is a decrease in mating ability in the third brood and the fact that we have eliminated females lacking sperms in their spermathecae, would not affect the basic conclusion that in *Culex pipiens fatigans* dominant lethal frequency is reduced by half in the third brood when compared to the first two broods.

ACKNOWLEDGMENT

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